THE EFFECT OF SIMULATED MICROGRAVITY ON ANTI-CMV AND ANTI-LEUKEMIA IMMUNE RESPONSES IN VITRO AND IN VIVO.

By

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To every giant whose shoulders I have climbed on, to stare into the abyss...

ABSTRACT

As the National Aeronautics and Space Administration (NASA), plans to return to the moon and send humans to Mars, crew health and safety concerns need to be examined with a focus on longer duration exploratory class missions. One of the transient changes that has been observed during the ISS missions is dysregulation of immune system. Latent viral reactivation and diminished cellular mediated immunity along with a TH2-cytokine shift being the consistently observed effects of spaceflight on immune system. However, most of the changes observed in spaceflight are a composite effect of stress, microgravity, radiation, circadian disruption, altered nutrition, and sleep disturbances, all of which have an immune-altering effect. Discerning precise effects of the various components of spaceflight become crucial to devise appropriate countermeasures. Ground-based simulated microgravity systems can be used to understand the specific effects of microgravity on human immune system. Rotary cell culture system (RCCS) is a NASA validated ground-based model to simulate microgravity. Using this quiescent low-shear stress environment, human immune cells can be exposed to simulated microgravity (SMG) for brief periods by randomizing the gravity vector and facilitating continuous free fall.

In short-term spaceflight, latent viral reactivation along with lowered viral-specific T cell function has been recorded. Healthy functioning of viral-specific T cells is a prerequisite to controlling viral infections. So, it becomes imperative to examine if microgravity plays a role in this reduction of viral-specific T-cell function.

There is also a paucity of data on effect of spaceflight and simulated microgravity on γδ-T cells. These crucial effector lymphocytes are considered a connecting link between innate and adaptive immune system. Their function in spaceflight, especially Vγ9Vδ2 T cell subset, becomes important to control hematological malignancies in early stages.

Therefore, this dissertation examined the effect of simulated microgravity on expansion potential and function of viral specific T-cells and γδ-T cells.

Exposure to SMG impaired in vitro expansion of CMV-specific T-cells (RM ANOVA, $F(1.571, 6.283)= 8.367$, $p=0.0198$). 10-million PBMCs at day1 of the expansion in SMG-exposed condition yielded 17.63±3.75 million (MEAN±SEM, N=5) CMV-specific T-cells at the end of the expansion. In comparison, STATIC-1G control cells expanded to 33.8±7.57 million, while 1G-rotational control exposed cells expanded to 28.32±7.21 million cells. However, exposure to SMG did not affect in vitro function of CMV-specific T-cells (RM ANOVA, $F(1.357,12.21)=0.7434$, $p=0.4457$). Hundred thousand cytotoxic (CD8+) CMV-specific T-cells exposed to SMG at the end of expansion killed 804.6±166.3 (MEAN±SEM, N=10) autologous PHA blasts pulsed with CMV peptides. In comparison, 1G-control and 1G-rotational control killed 909.2±160.6 and 669.7±125 PHA blasts respectively.

Exposure to SMG also impaired in vitro expansion of γδ-T cells (Wilcoxon signed ranks test, $p=0.039$). 10 million PBMCs at day 1 of the expansion in SMG-exposed condition yielded 102.3±23.07 million (MEAN±SEM, N=9) γδ-T cells at the end of the expansion. In

comparison, 1G-control PBMCs yielded 113.7±23.91 million γδ-T cells. Γδ-T cells that were exposed to SMG and later expanded in 1G showed downregulation of inhibitory receptor CD158b (paired t-test, p=0.03) and killed more U266 target cells (paired t-test, p=0.04) compared to γδ-T cells that were expanded in 1G. Exposure to SMG upregulated activating receptor NKG2D on γδ-T cells that were expanded in 1G (Wilcoxon matched-pairs signed rank test, p=0.0078), without concomitant increase in function. Exposure to SMG did not impair γδ-T cells' ability to kill tumor target cells (K562: paired t-test, t(8) =0.5032, p=0.628; U266: paired t-test, t(8) =0.1479, p=0.886).

Another limitation of spaceflight data is lack of *in vivo* functional data. Although we have observed decreased *in vitro* function of various immune cells in several flight and ground-based simulation studies, how this translates to a more physiologically relevant *in vivo* model remains to be explored.

Ergo, this dissertation also improved upon the current *in vitro* data by examining how exposure to SMG using a RCCS, affects *in vivo* anti-leukemia activity of human effector lymphocytes. A humanized NSG-tg(hu-IL15) mice model, which is used in a pre-clinical setting for hematopoietic stem cell transplantation studies, was used to examine the *in vivo* effect of exposure to SMG on PBMCs. Furthermore, we examined the efficacy of Zoledronic acid+IL2 (ZOL+IL2) therapy as a possible spaceflight countermeasure to revive the *in vivo* anti-leukemia activity of SMG exposed PBMCs. ZOL+IL2 is a clinical therapeutic strategy to accelerate favorable immune reconstitution. This therapy also improves NK cell and γδ-T cell

activity *in vivo*, after a hematopoietic stem cell transplantation. Therefore, we expected administration of ZOL+IL2 to abrogate the effect of exposure to SMG on human PBMCs, by stimulating NK cells and γδ-T cells.

Exposure to SMG impaired anti-leukemia activity of human immune cells in vivo. Tumor growth control was compared between mice that were injected with PBMCs exposed to SMG (TUMOR+SMG PBMCs) or 1G-control (TUMOR+1G PBMCs) to evaluate the effect of SMG on anti-leukemia activity of human immune cells in vivo. Mice injected with tumor cells only was used as a reference for unrestrained tumor growth. Bioluminescent intensity (BLI) score was used as a measure of tumor burden. A mixed effects model was used to analyze BLI scores with 'condition' (TUMOR control, TUMOR+SMG PBMCs, TUMOR+1G PBMCs) and 'time' as main effects and an interaction term 'condition*time' in the model. This revealed that tumor grew differentially over time in different conditions. A pairwise comparison revealed that 1G-exposed PBMCs controlled tumor growth better than SMG-exposed PBMCs ($p<0.001$). Peak BLI reached during the experiment further understated the inability of SMG-exposed PBMCs to control tumor growth (Friedman test, $p=0.0018$, $N=12$, TUMOR+SMG PBMCs>TUMOR+1G PBMCs). Exposure to SMG did not alter engraftment, survival or graft-versus-host-disease (GVHD) dynamics.

ZOL+IL2 therapy improved anti-leukemia activity of human immune cells in vivo. Mice that received SMG PBMCs and given ZOL+IL2 therapy controlled their tumor better compared to mice that received SMG PBMCs without ZOL+IL2 therapy (Mixed effects model, $p=0.0004$).

There were no differences in tumor control between mice that received SMG PBMCs along with ZOL+IL2 therapy and mice that received 1G-exposed PBMCs. This showed that ZOL+IL2 therapy abrogated the loss of in vivo function after exposure to SMG. ZOL+IL2 therapy did not alter survival and GVHD dynamics. There were non-significant increases in NK cell and γδ-T cell engraftment throughout the experiment in blood of the animals, showing that ZOL+IL2 therapy improved anti-leukemia effector immune cell engraftment, which helped in tumor control.

In summary, these experiments advance our understanding of the effect of simulated microgravity on immune cells. Exposure to SMG detrimentally affects immune cell function and expansion potential both in vitro and in vivo. These negative effects could impair crew health and performance during an exploratory class missions. These experiments also highlight the importance of microgravity as a contributor to the immune dysregulation observed in spaceflight. Future studies should explore the sustenance of this detrimental effect in confluence with other perturbations of spaceflight. Future investigations should also include relevant immunotherapeutic countermeasures to improve crew health and performance during long-duration exploration class missions.

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Chapter 1

Introduction

Space— ‘the final frontier’—has been a challenging arena for humans to conquer. Exploring beyond the protective blanket of earth’s atmosphere would involve facing hazardous challenges especially in long duration exploratory missions like returning to the moon, establishing lunar bases, asteroid exploration and reaching Mars. This would entail humans being exposed to multitude of hazardous extraneous factors like radiation, microgravity, partial gravities on lunar (0.16G) and Martian (0.37G) surfaces, circadian misalignment, altered microbial virulence, high G forces of launching and landing, and celestial dust (B. Crucian & Sams, 2009; Guéguinou et al., 2009; Rooney et al., 2019) which are collectively termed ‘space exposome’. In addition to these extraneous factors, intrinsic effect of spaceflight on human physiology, and its several organ systems, that have evolved for millions of years in 1G ($9.8 m/s^2$) gravitational force of the earth is a challenge that needs to be overcome before humans become multi-planetary.

Immune dysregulation has been reported in both short and long duration spaceflight. Short duration space shuttle missions usually last 14-17 days (B. E. Crucian, Stowe, Pierson, & Sams, 2008b; B. Crucian et al., 2013; B. Crucian, Stowe, Quiriarte, Pierson, & Sams, 2011; S. K. Mehta et al., 2014), and long duration missions to the International Space Station (ISS) usually last 5-6 months (B. Crucian et al., 2015; Satish K. Mehta et al., 2017). Major alterations to immune system observed include diminished cell mediated immunity, latent viral reactivation and TH-2 cytokine shift. However, limitations of spaceflight data are that the observed changes are a composite effect of ‘space exposome’ on human body. It is tough to discern the precise effect of each aspect of spaceflight on the human immune system, which would facilitate designing countermeasures. Processing time delays of ‘in-flight samples’ and

landing stress confounding results in 'post-flight samples' are other major limitations (B. Crucian et al., 2014b).

Another limitation of spaceflight immunology data is that most of the data was gathered from *in vitro* assays. Although *in vitro* assays help us to estimate the functional activity, how this translates to more physiologically relevant *in vivo* activities need to be explored. Tumor surveillance, homing, extravasation, tumor infiltration, and tumor lysis are some *in vivo* activities that can be evaluated using *in vivo* models. Humanized mice models can be used to estimate *in vivo* anti-leukemia activity of human immune cells.

Tumor transformation due to radiation exposure and latent viral reactivation due to physiological and psychological stress are two of the major concerns in relation to immune system during long-duration exploration class missions. Natural killer cells are a crucial set of innate immune cells that control early tumor growth (Chiossone, Dumas, Vienne, & Vivier, 2018). Previous studies have shown reduction in NK cell function during long duration spaceflight (Bigley et al., 2019). This reduction in NK cell activity appears to be more pronounced in first-time fliers to the ISS hinting at some adaptive response in veteran astronauts. We have previously shown that exposure to 12-hour simulated microgravity (SMG) also impaired NK cell killing against all the target cells (K562, U266, 721.221, 221.AEH) tested (fig. 1a-d) (Mylabathula et al., 2020). NK cells expressed lower perforin and granzymeB after exposure to SMG. This exposure also lowered levels of degranulation (CD107a+) and effector cytokine (TNFα and IFNγ) production when exposed to target cells (fig. 1e). Exposure

to SMG did not suppress K562 susceptibility to NK cell killing showing that cancer cells might be more resistant to effects of SMG compared to immune cells (fig. 1f). This might tip the balance in the favor of tumor transformation during a long duration mission with continuous radiation exposure and latent viral reactivation. These immune hazards further underscore the importance of understanding the effects of microgravity on various cell populations that control ‘tumor transformation’ and ‘viral reactivation’. Two of them—γδ-T cells and viral-specific T-cells—are being investigated in this dissertation.

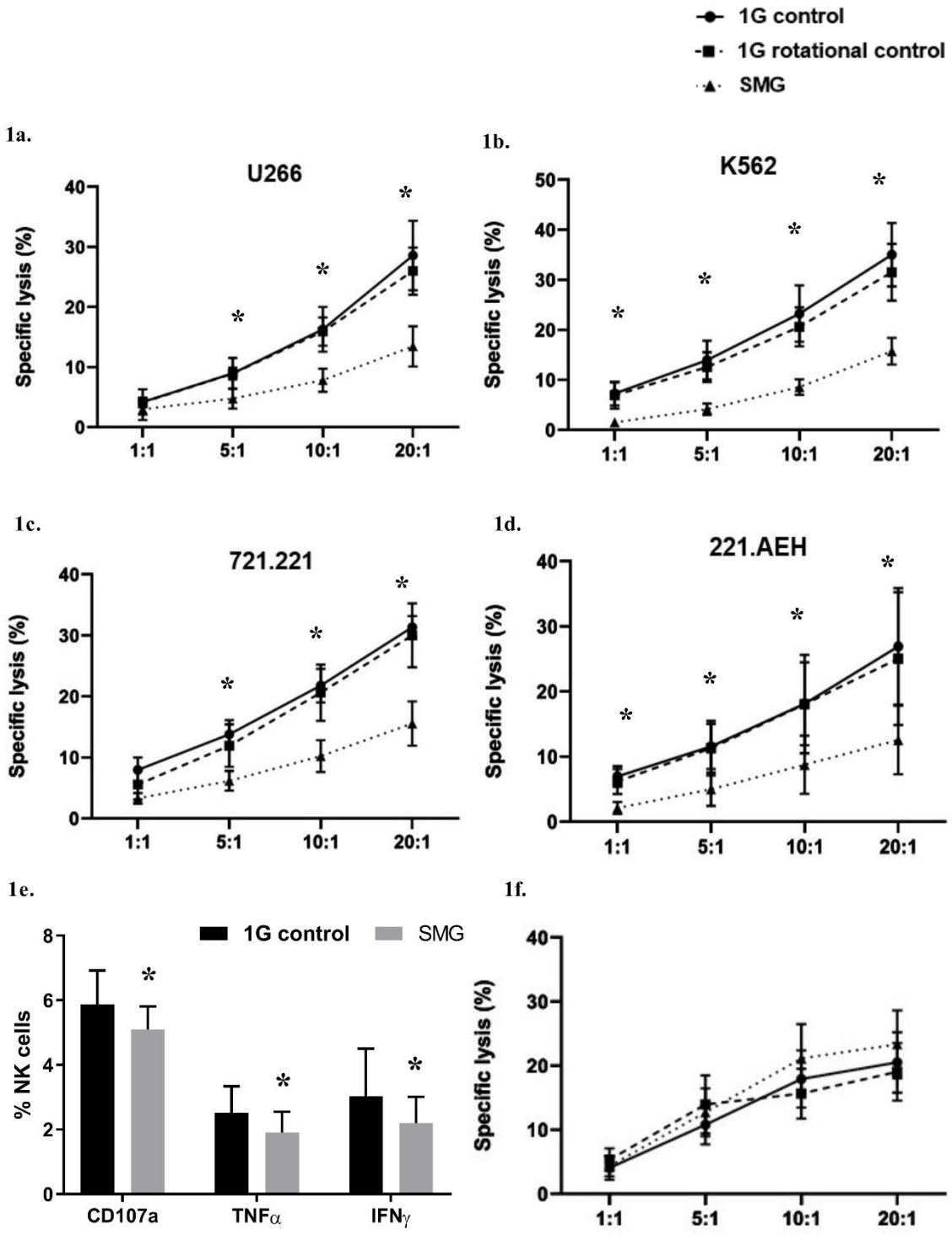


Figure 1. Effect of simulated microgravity on NK cell function. 1a-d. Exposure to SMG suppressed NK cell function (specific lysis) against four target cells (k562, U266, 721.221 and

221.AEH) 1e. Exposure to SMG suppressed NK cell degranulation and effector cytokine production when exposed to target cells later. 1f. Exposure to SMG did not affect K562 susceptibility to NK cell killing.

Immune dysregulation due to extreme environments is not an isolated occurrence in spaceflight. Similar immune perturbations occur in several spaceflight analogs, which hint at a contributory role that various factors play in contributing to the aggregate dysregulation observed in spaceflight. These analogs include isolation and confinement stress seen in winter-over Antarctica expeditions, Arctic Devon Island expeditions, NASA extreme environment mission operations (NEEMO); cephalad fluid shift seen in head-down bed rest (HDBR) studies and murine hind-limb unloading studies; short-term weightlessness like parabolic flight, sounding rockets, drop towers and simulated microgravity instruments like rotating wall vessels (RWV), 3D-clinostats, and random positioning machines. While the financial and logistical constrains have limited availability of human data from spaceflight, these analogs have been used to make scientific estimates of the effect of spaceflight on the immune system. Additionally, they can also be used to test efficacy of possible countermeasures to mitigate the risks of spaceflight. For example, vitamin D supplementation during Antarctica winter-over expeditions has shown to improve calcium homeostasis, and also reduce stress-induced Epstein-Barr virus reactivation (Zwart et al., 2011). Furthermore, these spaceflight analogs can also be used to identify the precise effect of each component of spaceflight. For instance, exclusive effect of microgravity on immune cells can be detected using SMG machines. Rotating wall vessels, when rotated on a horizontal axis, in a low-shear quiescent environment,

simulate the weightlessness of space by allowing the cells to experience continuous free fall and by randomizing the gravitational vector (Goodwin, Schroeder, Wolf, & Moyer, 1993; Klaus, 2001; Cheryl A. Nickerson et al., 2003; Schwarz, Goodwin, & Wolf, 1992). Using this NASA-validated RWV called rotary cell culture system (RCCS^{TM}), human immune cells can be exposed to SMG.

Therefore, here we propose an *in vivo* model to evaluate the effect of exposure to short term (12h) SMG on 'effector lymphocyte killing'. This will be accomplished by using NSG-tg(hu-IL15) mice that have been genetically manipulated to have an immature immune system. This facilitates easier colonization of the mouse body by human immune cells. The indigenously produced human IL15 in these mice will help NK cells thrive. Hence, by pre-exposing human PBMCs to SMG, we propose to estimate the effects of microgravity exposure on *in vivo* dynamics of NK cells as a measure of immune surveillance as well as cytotoxicity *in vivo*. We will also evaluate the effect of zoledronic acid+IL-2 therapy as a possible countermeasure to SMG induced loss-of-function. Additionally, we will use our 12h SMG exposure model to evaluate expansion potential, effector cytokine production and cytotoxicity of VSTs that are specific to CMV antigens and Vγ9/Vδ2 T cells. Since we see latent viral reactivation during spaceflight (B. Crucian et al., 2015; S. K. Mehta et al., 2014; Rooney et al., 2019), optimal functioning of viral-specific T-cells (VST) is a requisite. Since there is evidence of suppressed VST function (B. Crucian et al., 2013; S. K. Mehta et al., 2013) during short term spaceflight, there is a need to see if this translates to short term SMG (12h) exposure. This would help us decipher if there is a microgravity component to the loss-of-function in spaceflight.

Tumorigenic stimulus like ionizing radiation occurs in multiple forms during spaceflight. Radiation in space is composed of galactic cosmic radiation (GCR), solar particle events (SPE) and trapped radiation. Solar particle events like solar flares and coronal mass ejections inject the interplanetary space with high-energy protons, high-energy charged (HZE) nuclei and neutrons travelling at relativistic speeds. Trapped radiation zones like 'Van Allen belts' consist of radiation dense zones. Venturing beyond low earth orbit (LEO) will involve traversing these zones of trapped radiation. This also will contribute to fluctuations in the expected dose-rates during a 2-year mission. While definitive solutions to the 'radiation problem in space' will be a material sciences challenge involving sufficient shielding, other measures like nutritional, pharmacological and immunological countermeasures can offer complementary solutions from the life sciences branch. In this context, effect of microgravity on γδ T-cells becomes a very pertinent question to be answered. γδ T-cells are a sparse but important subset of effector T-lymphocytes that inhabit the gut lining, mucosa and blood compartments. They have multiple activating and effector pathways that mimic both T-cells and NK cells. Therefore, they are considered a connecting link between innate and adaptive arms of the immune system. They work hand-in-hand with NK cells to suppress tumorigenic growth in its initial stages before the adaptive immune system can gear up. There is a lack of γδ T-cell data in the spaceflight niche. In this dissertation, we will examine if exposure to short-term SMG will reduce γδ T-cell ability to expand and later kill cancer cells in an *in vitro* setting. We will also use systemic administration of zoledronic acid to expand γδ T-cells *in vivo* to augment anti-leukemia activity.

Chapter 2

Literature Review

There are various epistemic uncertainties that confound any projections about the response of human body on a mission to Mars that is estimated to last two years. Nevertheless, valuable data relating to human spaceflight in low earth orbit (LEO) has been gathered using the national laboratory on the International Space Station (ISS) over the last few decades. This literature review will discuss the effect of spaceflight on human body, and then more intricately discuss the current understanding of the effect of spaceflight on the human immune system, and results from various analog modules.

Maintenance of crew health and performance are of paramount importance for the success of the mission. Current 6-12-month missions to the International Space Station (ISS) have suggested that human body can tolerate the physiological challenge of short-term spaceflight with a few permanent and many temporary decrements. Hence, the idea of a 'space normal'—a new acceptable state of homeostasis—for spaceflight has been posited.

1. Spaceflight physiology

Ever since the advent of spaceflight, investigations have been conducted on how spaceflight affects human body. In the words of the first human in space, Yuri Gagarin, "The feeling of weightlessness was somewhat unfamiliar compared with Earth conditions. Here, you feel as if you were hanging in a horizontal position in straps. You feel as if you are suspended"(Siddiqi 1966-, 2000). This feeling of suspension is a novel stimulus to a human body. This was the dawn of the era of human spaceflight.

While a 2-lead ECG, pulse, and pneumogram were the only biomedical data collected from Gagarin's flight, current ISS missions contain equipment to measure everything from a 12-lead ECG to biochemical analysis of blood and urine. Many studies conducted on ISS have increased our understanding of alterations in normal biological processes. These data can be used to make relevant predictions for a 2-year mission to Mars.

Crucial concerns that can affect mission success for a Mars flyby and landing currently include space radiation and intracranial hypertension (ICH). Mission relevant dose exposure to simulated galactic cosmic radiation (GCR_{sim}) has shown neurocognitive defects in murine models (Acharya et al., 2019; Britten et al., 2014; Parihar et al., 2016). Exposure to GCR will also have various long term effects including cancer (Cucinotta, 2014; Cucinotta, To, & Cacao, 2017), CNS degeneration (Blue et al., 2019; Cekanaviciute, Rosi, & Costes, 2018; Chancellor, Scott, & Sutton, 2014), radiation induced cardiovascular disease (RICVD) (Sylvester, Abe, Patel, & Grande-Allen, 2018). Intracranial hypertension leads to various structural changes in the brain. Recent studies have shown increased lateral and third ventricular volume (Van Ombergen et al., 2019), vertical displacement of the brain, changes in volume of the central sulcus, narrowing of cerebrospinal fluid spaces at the vertex (Roberts et al., 2017) and decrease of gray matter in orbitofrontal and temporal cortexes (Van Ombergen et al., 2018). Both these studies showed significant effect of time on the magnitude of these structural changes. Therefore, these structural changes can be expected to progress further during a 2-year mission to Mars causing major neurocognitive deficits. Van Ombergen's study included a long-term follow up, which showed sustained volume changes 7-months after a long-term ISS mission hinting at consequences that might extend beyond the mission duration. Cephalad fluid shift

also causes spaceflight associated neuro-ocular syndrome (SANS or VIIP) (Zhang & Hargens, 2018), resulting in permanent visual acuity deficits. Other major concerns include renal calculi formation due to calcium excretion (Liakopoulos, Leivaditis, Eleftheriadis, & Dombros, 2012; Smith et al., 2015), altered immune response (B. Crucian et al., 2015; Rooney et al., 2019), altered host-microorganism interactions and microbial virulence (Foster, Wheeler, & Pamphile, 2014; Senatore, Mastroleo, Leys, & Mauriello, 2018). Minor concerns include perturbations that might not affect mission success but might affect optimal crew performance. They include sensorimotor alterations (Mulavara et al., 2018; Wood, Loehr, & Guilliams, 2011), reduced muscle mass and strength (Maffiuletti, Green, Vaz, & Dirks, 2019; Narici & de Boer, 2011), reduced aerobic capacity (Ade, Broxterman, & Barstow, 2015), and decreased bone mineral density resulting in fractures (Swaffield, Neviaser, & Lehnhardt, 2018). Other concerns include orthostatic intolerance (Shen & Frishman, 2019), sleep loss and circadian misalignment (Brainard, Barger, Soler, & Hanifin, 2016; Wu et al., 2018).

2. Spaceflight Immunology:

While immune suppression might lead to increased infectious diseases, altered wound healing, latent viral reactivation, increased tumor transformation potential and disease susceptibility, an overactive immune system might lead to allergies, hypersensitivity, autoimmune diseases. Therefore, a perfect balance of the immune system is essential for a healthy living.

Dysregulation of the immune system during spaceflight is especially precarious given the limited exploration class medical capabilities that can be expected during a multiple year mission to Mars (Antonsen, E, Bayuse, T, 2017; Blue et al., 2019). A simple medical event on

earth like a latent CMV reactivation might be threatening mission success during spaceflight. A crew member with already weakened/overburdened immune system might progress from simple skin lesions to ocular CMV manifestations like endothelitis, which is rarely seen in immunocompetent hosts (Joye & Gonzales, 2018). When this is coupled with modified pharmacokinetics and pharmacodynamics in microgravity resulting in altered efficacy of drugs (Kast, Yu, Seubert, Wotring, & Derendorf, 2017), medical treatments like topical or oral ganciclovir might not be sufficient. Additionally, we have to consider that while direct contact is necessary for transmission of CMV on earth, in microgravity, there might be increased routes of transmission (for example via atmospheric routes through ocular or nasal openings). Several factors involved in spaceflight are known modulators of the immune system (figure 2). The overall magnitude of each of these factors will depend on the length and the directive of the mission.

15 out of 29 Apollo astronauts developed bacterial or viral infections whether during or after the 5 to 12-day missions (Johnston et al., 1975). With minimal amount of biomedical data collected during these missions, post-flight leukocytosis (mostly driven by neutrophilia) was observed but lymphocyte reactivity results remained inconclusive (Fischer, Daniels, et al., 1972). Immunoglobulin levels showed an elevation of I_gA and α2-globulin while I_gM, I_gG and I_gD remained unchanged (Fischer, Gill, et al., 1972). These signs of an 'acute phase reaction' was a sign of pro-inflammatory status, most probably a result of psychological and physiological stress response. While the Apollo astronauts showed no elevation in plasma cortisol levels, a 24% increase in urinary cortisol levels was observed in post-flight samples (Johnston et al., 1975). Three manned Skylab missions of 28, 59 and 84 days duration showed

elevated plasma and urinary cortisol levels in the post-flight samples ("BIOMEDICAL RESULTS OF SKYLAB," 2013). Skylab experiments were also the first to hint at reduced cellular immunity. Blastoid transformation of lymphocytes in response to PHA was markedly decreased in all the missions irrespective of duration and recovered within 7-days of returning to earth ("BIOMEDICAL RESULTS OF SKYLAB," 2013). No in-flight samples were collected during Apollo and Skylab missions.

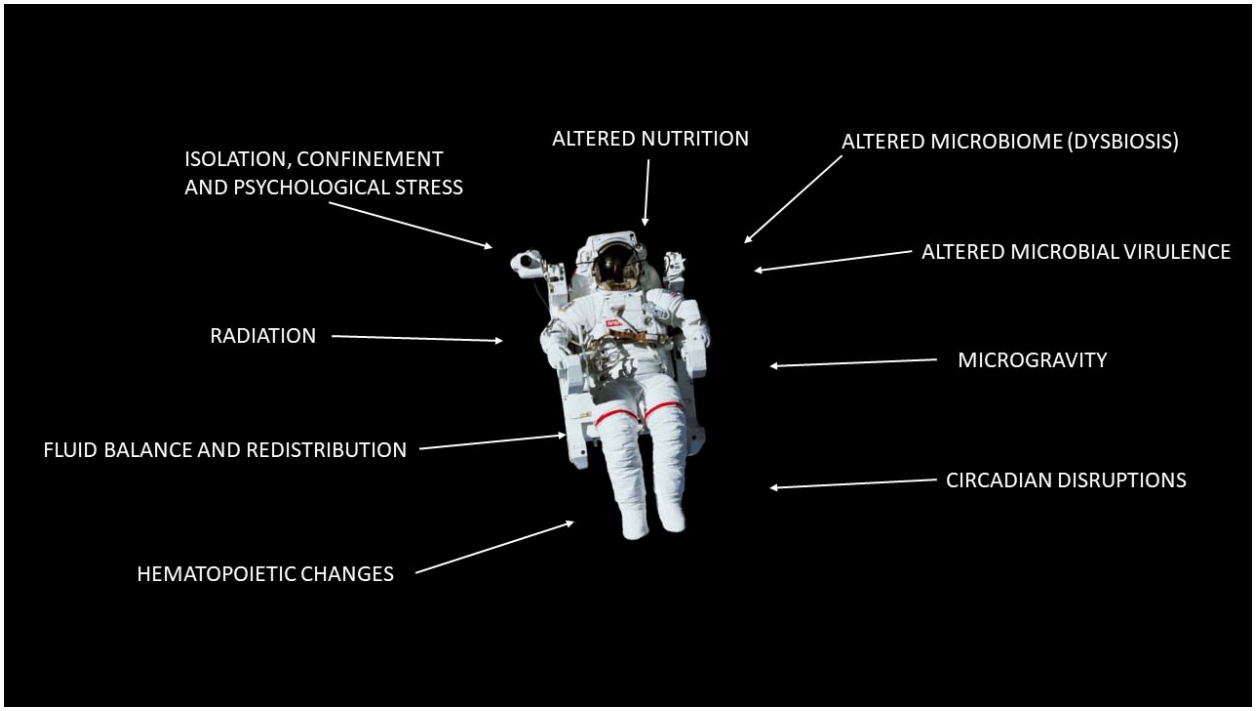


Figure 2. Various factors of spaceflight that could affect the immune system.

Shuttle missions also showed reduced cellular immunity, with in-flight and post-flight T-cells showing reduced early activation (CD69+) and late activation (CD69+ CD25+) but only when stimulated with staphylococcal enterotoxin A and B (SEA and SEB) (B. Crucian et al., 2013).

No such reduction in activation was observed when anti-CD3 and anti-CD28 antibodies were used to stimulate the T-cells suggesting that while non-specific stimulation with a mitogen (PHA) might be affected, T-cells retained the cellular mechanisms to be specifically stimulated with stimulants that are more powerful. This suggests that either the 'activation threshold' for T-cells might be elevated during spaceflight or the antigen presenting cells are necessary for activation when stimulated with superantigens (SEA and SEB). Shuttle missions also showed that during flight, WBC major subsets did not alter while CD8 T-cell subsets showed fluctuations. Expansion of cytotoxic CD8+ T-cells (CD28+/CD244+) was observed 10 days prior to and during the mission. This might be in response to the latent viral reactivation and stress. The crewmembers are under considerable stress prior to launch and immediately after docking with the ISS. 53% (47/89) of the crew members that flew on the shuttle missions shed at least one of the three (CMV, VZV and EBV) latent viruses (Rooney et al., 2019). This is mostly understood to be a stress response as significant increases in CORTISOL:DHEA molar ratio (area under the curve) were observed in saliva of in-flight samples compared to pre- and after flight (Rooney et al., 2019). However, Stowe et al. reported elevation of urinary cortisol and norepinephrine in post-flight samples but this was not limited to crewmembers with EBV reactivation. Interestingly, the urinary cortisol levels did not differ between astronauts with and without EBV reactivation but epinephrine and norepinephrine levels were higher in the EBV-reactivating group compared to those with reactivation (Stowe, Pierson, & Barrett, 2001). This suggests an activation of sympathetic-adrenal-medullary axis while absence of activation of hypothalamo-pituitary-adrenal axis. No in-flight samples were collected for this study and the reported elevations in stress hormones were from R+0 (day-of-return) samples. Therefore, it is necessary to extricate the effect of landing stress from these results. Another important

finding from the shuttle crewmembers is reduced function of viral specific T-cells (CMV and EBV), seen as reduced intracellular IFNγ production after stimulation with specific peptides. This dissertation project involves expanding CMV-specific T-cells and exposing them to SMG and later evaluating their IFNγ production and cytotoxicity. This will delineate whether microgravity plays a contributory role in this loss of function. Agha et al. reported a correlation between pre-flight fitness status and maintaining fitness during a long-term mission with reduced incidence of latent viral reactivation (Agha, Mehta, et al., 2020). Another noteworthy observation was a widespread reduction in cytokine production (IFNγ, TNFα, IL10, IL4, IL5 and IL6) when in-flight PBMCs were stimulated with PMA+ ionomycin.

Effect of short-term spaceflight (Shuttle missions) on innate immune system is not so clear. Crucian et al. (B. Crucian et al., 2013) reported that monocytes retained their function and that in-flight monocytes when stimulated with LPS, not only secreted comparable levels of all cytokines measured but higher levels of IL-8 compared to L-180 (180 days prior to launch). Kaur et al. (Kaur, Simons, Castro, Ott, & Pierson, 2005) reported reduced phagocytosis, oxidative burst capacity and degranulation in monocytes collected on day of return (R+0). Studies about neutrophil function with short duration spaceflight seem ambiguous. While Kaur et al. (Kaur, Simons, Castro, Mark Ott, & Pierson, 2004) reported reduced neutrophil function (oxidative burst capacity) only in 9-11 day missions but not in 5-day missions; Stowe et al. showed a 10-fold decrease in chemotaxis (Stowe et al., 1999).

Long term spaceflight (usually 6-month) missions to the ISS showed that adaptive immune dysregulation continued to persist (B. Crucian et al., 2015). Differential activation responses to various stimulants, seen in short-term spaceflight continued with in-flight samples in long-duration spaceflight. Both CD4+ and CD8+ T-cells showed reduced early (CD69+) and late (CD69+/CD25+) activation with SEA and SEB only in early flight (day 14) time point. Similar to short-term spaceflight, this reduced activation was not seen with more potent anti-CD3 and anti-CD28 stimulation. Furthermore, stimulation with PMA+ ionomycin showed lower percentage of CD4+ T-cells producing IL-2 at R+0 (day of return). Interestingly, viral-specific T-cell function seems to have recovered from loss-of-function observed during short-term spaceflight. PBMCs continued to produce lower amounts of IFNγ, IL4, IL5, IL10, IL17A, TNFα and IL6 when stimulated with PMA+ ionomycin. While B-cell homeostasis and function were maintained during spaceflight (Spielmann et al., 2019), NK cell function seems to be reduced in-flight (Bigley et al., 2018). Natural killer cells are effector lymphocytes that do not undergo thymus mediated antigen education and instead use their signaling amplitude on MHC ligands as identity checks to eliminate virus-infected cells and malignantly transformed cells. Therefore, when both these immune challenges (latent viral reactivations and increased carcinogenic exposure via the 'space exposome') are exacerbated during long duration spaceflight, healthy functioning of NK cells is a prerogative. NK-cells isolated from PBMCs collected during mid-flight time point (flight day 90) lysed 50% fewer k562 cancer cells compared to age and sex matched controls. This suppression in NK cell function seems to be more pronounced in first-time fliers compared to crew members with prior flight experience, suggesting some blunting of this effect after acclimatization to spaceflight. Whether this is related to different stress levels remains unclear since there was no evidence suggesting lower

stress hormone levels in “veteran” crewmembers. Like with all spaceflight data, low subject numbers, time delays in processing and scarcity of subjects (one or two subjects every 6 months) make collection of sufficient data cumbersome.

While reductions in NK cell function might hint at weakened innate immunity during spaceflight, Agha et al. recently showed that salivary antimicrobial protein levels increased during spaceflight suggesting that innate immunity might be intact if not enhanced (Agha, Baker, et al., 2020). However, this might be in response to latent viral reactivation observed in the astronauts. While salivary cortisol levels remained normal compared to ground-based controls, higher cortisol levels were observed in viral shedders compared to non-shedders. This suggests that stress response to spaceflight might not be universal and that there might be a personalized component to the perception of the stress of spaceflight.

Elevations in cortisol:DHEA were also observed corroborating the previous data but this appears to be driven by reductions in DHEA levels than increase in cortisol levels. While both these hormones are produced by the adrenal glands, they are supposed to have an inter-modulatory effect on each other. High DHEA levels are supposed to reduce cortisol levels and antagonize most of the immune-suppressive effects of cortisol. This is another mechanism through which medium and short term stress is supposed to be modulated by the psycho-neuro-immunological network to maintain homeostasis (Kamin & Kertes, 2017). Buchheim et al. also reported that diurnal salivary cortisol levels remained steady in Russian cosmonauts (J. Buchheim et al., 2019). Interestingly, there were evidences of activation of the

endocannabinoid system, represented by elevation of anandamide (N-arachidonylethanolamine) at late in-flight time point (flight day 150). Elevation of anandamide is also considered a stress response, that is supposed to have strong immunomodulatory effects (J. I. Buchheim et al., 2018). Therefore, taken together, these studies show an effect of stress response from spaceflight affecting the immune system. However, the magnitude of this effect seems to vary along with high amount of interpersonal variability.

Understandably, many factors including prior flight experience, training, workload, sleep schedules, team cohesion, exercise training regimen, nutritional preferences, and mission goals all play a contributory role to how stress is perceived and handled which ultimately affects the immune system.

3. Spaceflight analogs

'Stress' can be defined as a physiological response of an organism to an unfavorable stimulus from the environment. Human physiology responds to stress by releasing hormones that have a profound effect on the immune system. In the initial seconds, the sympathetic nervous system activates the adrenal medulla, which releases epinephrine and norepinephrine. This connection is termed sympathetic-adrenal-medullary (SAM) axis. Later, hypothalamus releases corticotropin-releasing hormone (CRH) that acts on the anterior pituitary to release adrenocorticotropic hormone (ACTH) that acts on the adrenal cortex to release cortisol. This connection is termed the hypothalamo-pituitary-adrenal (HPA) axis. All these hormones have an immuno-modulatory effect. Since stress is thought to play a huge role in immune system

dysregulation observed with spaceflight, using terrestrial models that can replicate the stress can be used as analogs to study effects of stress on human immune system. Supportive evidence for this can be found in early studies conducted by Glaser (Glaser, Kiecolt-Glaser, Speicher, & Holliday, 1985; Glaser, Kiecolt-Glaser, Stout, et al., 1985) and Gleeson (Gleeson et al., 2002)et al. Immune dysregulation similar to that found in astronauts was found in highly stressed cohorts. Elite swimmers during intense training have shown EBV reactivation and shedding. Medical students during examinations and military cadets have shown signs of suppressed cell mediated immunity along with EBV, CMV and HSV-1 shedding.

Antarctica winter-over expeditions:

Amongst all the human spaceflight simulation analogs, it is probably Antarctica winter-over studies that show most immunological similarities to spaceflight. Sever temperatures, 3-months of complete darkness, high altitudes, isolation and extreme remoteness making evacuations impossible make Antarctica winter-over stays psychologically extremely stressful. Diminished cell-mediated immunity along with Epstein-Barr virus reactivation were observed in Antarctica expeditioners by Mehta et al. (Satish K. Mehta, Pierson, Cooley, Dubow, & Lugg, 2000). Expeditioners showed reduced delayed-type hypersensitivity (DTH) reaction, a measure of cell-mediated immune response. A later study by Feuerecker et al. (Moreels, States, Ulrike, Quintens, & Sams, 2019) used a modified 'new global immunity test' assay that replaced the traditional DTH, also showed reduced cellular immunity. Response of whole blood to a mixture of bacterial, viral, and fungal antigens showed lower levels of TNFα, IL2, IFNγ, and IL10. This confirmed lower levels of cell-mediated immunity in Antarctica winter-

over expeditioners. A more recent study by Feuerecker (Feuerecker et al., 2019) showed that a 1-year Antarctica stay at the Concordia station showed high levels of immune activation evidenced by elevated cytokine responses. This yearlong study might become very relevant in expecting what might occur in exploration class missions, as this might be hinting at response of immune system to chronic stress. However, genome transcriptional analysis also revealed hypoxic pathways being activated, which might be a confounding factor, as this might not occur in spaceflight. Chronic stress resulting in overactivation of immune system for long periods might also result in 'immune exhaustion' and 'inflammaging' in astronauts. Humoral immunity appears to stay intact during Antarctica winter-over expeditions (Shearer et al., 2001).

NASA Extreme Environment Mission Operations (NEEMO):

NASA has a laboratory 3.5 miles offshore the Florida Keys, at 60 feet depth. 14-day undersea deployments are supposed to elicit some amount of stress response. Hyperbaric hyperoxia in these habitats might not simulate spaceflight environment. However, it could represent extra-vehicular activities (EVAs) that occur in hyperbaric hyperoxia. Nevertheless, isolation, confinement and stress experienced by aquanauts might provide us with some hints about spaceflight. Strewe et al. (C. Strewe et al., 2015) found that as the 14-day mission progressed, there was evidence of leukocytosis, driven by neutrophilia and monocytosis, but with concomitant lymphopenia. No significant differences in cytokine production were observed. Innate immune system disturbances were observed with granulocytes showing higher

activation levels (β_2-integrin CD11b) and shedding of adhesion molecules (CD62L), hinting that these stress levels are enough to transform the innate system to a 'high alert' status.

Head-down bed rest studies:

Head down bed rest (HDBR) studies are used as spaceflight analogs to simulate the cephalad-fluid shift seen in spaceflight. This analog simulates musculoskeletal decrements but does not stimulate the effects of microgravity and stress. Therefore, some studies, for example Crucian et al. (Sams et al., 2009) have failed to show the latent viral reactivation and immune activation deficiencies that occur during spaceflight. There were also no significant cortisol changes suggesting microgravity and stress to be possible causes of immune dysregulation in this 90-day bed rest trial. Contrastingly, another study by Chouker et al. (Choukèr et al., 2001) argued that there was a psychic stress component to a 6° head down bed rest showing increases in 'current stress test' questionnaire along with increased urinary dopamine and norepinephrine excretion. This study also reported decreased CD4:CD8 ratio and increased IL6 plasma concentration. It should be noted that this was a 120-day trial. Therefore, restricted body movement for such extended periods might be causing hypokinesia-associated psychological stress to some extent. Kelsen et al. (Kelsen et al., 2012) showed suppression in cell-mediated immunity following a 21-day head down bed rest. Reduced production of IL2, IFNγ and TNFα was observed during bed rest when stimulated with PHA. This study also failed to notice any latent viral reactivation. Another study (Stowe, Yetman, Storm, Sams, & Pierson, 2008) by Stowe et al. showed spaceflight resembling patterns of urinary epinephrine and cortisol excretions in just 14-day head down bed rest. In this study, subjects were exposed to G-forces

of launching and landing using a human centrifuge to mimic Shuttle launch and landing patterns. Therefore, this could serve as evidence that the high G-forces might be responsible for urinary catecholamine excretion. This study also provided evidence for landing stress induced neutrophilia usually observed in return samples. Simulation of landing G-forces showed similar patterns of leukocytosis mainly driven by neutrophilia. A recent 45-day HDBR study by Xu et al. (Xu et al., 2013) showed decreased cell-mediated immunity, increased secretion of pro-inflammatory cytokines (IL1β and IL18) by B-cells and myeloid cells and reduced secretion of IFNγ and IL17 by activated T-cells. This study also showed increased serum cortisol during the bed rest. These studies taken together indicate that if psychic stress is a constituent of head down bed rest, it could mildly simulate spaceflight without latent viral reactivation.

Hind limb unloading studies:

Hind limb unloading studies, where rats or mice are hoisted up by their hind limbs to produce a 30° head-down tilt is used as a model to simulate the cephalad fluid shift seen in spaceflight (Morey-Holton & Globus, 2002). This model elicits similar stress patterns like spaceflight since a quadruped prey animal is restrictively hung by a support. Like HDBR studies, this was originally intended to study muscle and bone loss, but stress hormones (glucocorticoids and catecholamines) released show a hypothalamic oxidative stress (Sarkar et al., 2008). Pro-inflammatory response (increased IL1β, IL6) coupled with diminished cellular cytokine response (reduced IL2 production) (Felix et al., 2004) show similarities with humans upon return from spaceflight (R+0) (B. Crucian et al., 2014a). However, the results might be

signaling a systemic inflammatory response in the rodents. There is evidence of bacterial endotoxin in blood during hind limb unloading (Rivera, Tcharmtchi, Mendoza, & Smith, 2003). Excessive hydrostatic fluid pressure in the cephalad portion of body results in disruption of gut lining causing a 'leaky gut syndrome', allowing lipopolysaccharide (LPS) to enter the blood stream, which might be causing the systemic inflammation. Therefore, puritanical comparisons of hind limb unloading to spaceflight must be done with caution. Other additional factors like radiation have been reported to amplify detrimental effect of hind limb unloading on the immune system. For example, solar particle event (SPE)-like radiation has been shown to decrease bacterial clearance (M. Li et al., 2014). Hind limb unloading has also shown to increase tumor growth in mice (Lee, Ding, Kulkarni, & Granstein, 2005). Therefore, this analog model can also be used to evaluate effects of other factors on an already taxed immune system.

Parabolic flight:

Parabolic flight or 'vomit comet' is a peculiar flight pattern that involves near vertical nose-dive and nose-lift of a commercial jet. During the nose-dive, accelerating downwards at 1G ($9.86 m/s^2$) neutralizes the gravitational vector allowing the flight to experience near 0G for approximately 20 seconds during each injection. Therefore, this provides a great opportunity for conduction, convection, and signal transduction studies to be performed, although for very brief periods. Commencing the experiment at the beginning of injection into the downward parabola and fixing the cells at the end of it facilitates observing cellular interactions in real microgravity. While gravitational stress of parabolic flights affects human immune system,

stress response to altered gravity seems more complex. While some studies like Stervbo et al. (Stervbo et al., 2018) showed no changes in cortisol levels, other studies Strewe et al. showed a correlation of 'perceived stress' and signs of motion sickness with elevations in cortisol (Claudia Strewe et al., 2012). Interestingly, participants that did not show signs of motion sickness or elevations in cortisol, showed activation of the endocannabinoid system with elevations in 2-Arachidonoylglycerol. Similar elevations in another endocannabinoid, anandamide (N-arachidonoylethanolamine) were observed in cosmonauts (J. Buchheim et al., 2019) during ISS missions. Parabolic flights simulating microgravity, showed reduced delayed type hypersensitivity reactions and altered cytokine production in mice spleen (Kita, Yamamoto, Imanishi, & Fuse, 2004) mirroring spaceflight alterations. Neutrophils showed increased cytotoxic capabilities (I. Kaufmann et al., 2009) in humans, but *ex vivo* supplementation of adenosine reduced tissue toxic H_2O_2 production of the neutrophils via A2(A) receptor (Ines Kaufmann et al., 2011). This strategy might be helpful to prevent G-stress induced overactivation of immune system when landing on Mars surface.

Simulated microgravity:

Rotating wall vessels (RWV) and random positioning machines (RPM) have been used for decades to study effects of simulated microgravity. This effect was studied on various cells and tissues (Goodwin, Prewett, Wolf, & Spaulding, 1993; Goodwin, Schroeder, et al., 1993; Hammond & Hammond, 2001; Jessup, Goodwin, & Spaulding, 1993; Klaus, 2001; Monici et al., 2006; Schwarz et al., 1992; Tsao, Goodwin, Wolf, & Spaulding, 1992; Wuest, Richard, Kopp, Grimm, & Egli, 2015). Initially borrowing from botanists that used similar concepts to

study gravitropic effects on plant growth, NASA devised this system to culture cells and tissues in the microgravity of space. Soon realizing that these instruments can be used on earth to simulate microgravity, they have been used by life scientists to study the effects of simulated microgravity on Earth. Now, modeled microgravity is used in a wide variety of applications including tissue regeneration, 3D-cell culturing, tissue-cell interactions, host-pathogen interactions (Aleshcheva et al., 2016; Grimm et al., 2014; Higginson, Galen, Levine, & Tennant, 2016; Manley & Lelkes, 2006; Cheryl A. Nickerson et al., 2003; Wuest et al., 2015). Rotating wall vessel operates by suspending cells in a high-aspect (radius-to-depth) ratio vessel in a quiescent low-shear stress environment. When rotated on a horizontal axis at enough speed while maintaining laminar flow of the fluid, the cells can be subjected to randomization of gravity vector and continuous free fall. When this occurs over extended periods, the average gravitational vector reaches to a magnitude of 10^{-2}G. The aggregate force experienced by any cell in systemic physiology inside a human body —especially a motile cell, like most cells in the immune system—is dependent on multiple factors. These include hydrostatic forces of blood or extracellular fluid, tissue tensile strength, rigidity of extracellular matrix etc. Nevertheless, simulated microgravity machines have been accurately replicating the effect of microgravity on cells (Freed et al., 1999; Hammond & Hammond, 2001; Klaus, 2001; Lappa, 2003; Martinez et al., 2015; Walther, 2001).

In systemic physiology, simulated microgravity has shown effects on humoral responses (Fitzgerald et al., 2009), signal transduction (Paulsen et al., 2010), cell cycle (Thiel et al., 2012), and DNA repair mechanisms (Degan et al., 2005; Kumari, Singh, & DuMond, 2009; Moreno-villanueva et al., n.d.). Effect of simulated microgravity on lymphocytes include

alterations in proliferation (Martinelli et al., 2009), signaling pathways (Licato & Grimm, 1999), activation and function (Fitzgerald et al., 2009; Risso et al., 2005), lymphocyte locomotion through type 1 collagen (Pellis et al., 1997; Sundaresan, Risin, & Pellis, 2002), epigenetics (Singh, Kumari, & DuMond, 2010). Other major effects on the immune system include alterations in cytoskeletal structure, (Zheng et al., 2011), function of hematopoietic stem cells (D. Cao et al., 2019) and monocyte function (Yu, Zheng, Xiong, Cai, & Wang, 2011).

Buravkova et al. reported that NK cells retained their ability to recognize, maintain contact and lyse k562 target cells onboard the ISS compared to ground based controls (Buravkova, Rykova, Grigorieva, & Antropova, 2004) but this experiment was only performed twice. In contrast, Li et al. (Q. Li et al., 2013) used expanded NK cells to show 48-hour exposure to SMG impairs NK cell cytotoxicity, increases NK cell apoptosis, reduces expression of IFNγ by downregulation of NKG2D and NKG2A. However, expanded NK cell functional activity is not regulated by its phenotypic markers (Lieberman et al., 2018) and their potency/loss-of-function cannot be estimated as a consequence of receptor modulation. Our experiments with primary, naïve unexpanded NK cells show that even short-term exposure (12 hours) to SMG affects primary NK cells. NK cells lysed ~50% fewer cancer cells when tested in 16 subjects in four different cancer cell lines (k562, u266, 721.221, and 221.AEH). This reduction in function was due to reduced expression of cytotoxic granules, perforin and granzyme B. Primary NK cells also showed reduced degranulation (CD107a+), TNFα and IFNγ production when encountering target cells. Therefore, it is still not certain whether NK cells retain their function in microgravity. We propose to explore if this loss in function will extend to more

physiologically relevant *in vivo* model. Future *in vitro* and murine experiments onboard the space station could help answer this important question.

4. Spaceflight countermeasures

Countermeasures currently being considered include screening, nutritional, behavioral (exercise, positive experiences), and pharmacological interventions (B. E. Crucian et al., 2018).

Multiple *in vitro* remedies/countermeasures to SMG induced suppression of function have been successfully reported. These include interleukins (Q. Li et al., 2013), polysaccharides (Huyan et al., 2014), nutritional nucleotides (Hales et al., 2002; Kulkarni, Yamauchi, Sundaresan, Ramesh, & Pellis, 2005; Yamauchi et al., 2002), PMA stimulation (Simons, Gardner, & Lelkes, 2009), morin sulphates/glucuronides (Hsieh, Chao, & Fang, 2005), rhodiola rosea (Xu et al., 2016). However, the clinical efficacy, safety and drug interactions of these compounds remain untested.

ZOLEDRONIC ACID:

Zoledronic acid is an amino-bisphosphonate that has multiple clinical therapeutic applications including prevention of osteoporosis (Fobelo Lozano & Sanchez-Fidalgo, 2019), bone loss in cancer induced osteopenia (Mei, Xiang, Yang, & Xiang, 2019). Since bone mineral density loss due to gravity unloading in space is a major complication, life scientists are now considering zoledronic acid as a spaceflight therapeutic countermeasure (Cavanagh, Licata, &

Rice, 2005). Coincidentally, zoledronic acid also has many immunological consequences. One of the ways zoledronic acid improves bone mineral density is by inhibiting osteoclastogenesis by downregulating nuclear factor kappa-B ligand (RANKL). Zoledronic acid also improves anti-tumor immunity through suppression of T_{regs} which use the same RANKL ligand (Liu et al., 2016; Salaroglio et al., 2015). Tumor infiltration by T_{regs} promotes tumor evasion (Wilke, Wu, Zhao, Wang, & Zou, 2010). Suppressing T_{regs} activity improves anti-tumor activity by making the tumor microenvironment more accessible to cytotoxic (CD8+) T-cells, NK cells and tumor-associated macrophages (Aldinucci, Borghese, & Casagrande, 2019). Another immune cell type that zoledronic acid has an effect on are γδ-T cells. Zoledronic acid sensitizes tumors to γδ-T cell killing by increasing phosphoantigen expression on their surface (Caccamo et al., 2008; Hamilton, Clay, & Blackwell, 2011). Zoledronic acid is also used to expand γδ-T cells *in vitro* (Kondo et al., 2011). Antigen presenting cells take up zoledronic acid from the surrounding media and convert it to isopentenyl pyrophosphate (IPP). When they present this IPP to γδ-T cells, they are activated and in presence of IL2 expand to yield highly pure γδ-T cell product. This has been used as a strategy to expand γδ-T cells *ex vivo* for immunotherapeutic purposes. Zoledronic acid has also been shown to expand γδ-T cells *in vivo* (Monkkonen et al., 2007). Therefore, we propose to use zoledronic acid to revive anti-tumor activity by suppressing $T_{regs,}$ and activating and expanding (in the presence of IL2) γδ-T cells *in vivo*. We hope this will override SMG induced immune-suppression in an NSG-tg(hu-IL15) mice model.

IL-2 THERAPY:

Administration of IL-2 has shown to improve anti-tumor activity in pre-clinical mice models (Cheever, Greenberg, Fefer, & Gillis, 1982; Mazumder & Rosenberg, 1984) by activating lymphocytes into lymphokine activated killer (LAK) cells. It has also been shown to be an effective immune-therapeutic agent against various cancers in clinical trials by improving NK cell function (Kimpo, Oh, & Lee, 2019; Muntasell et al., 2017; Rosenberg, 2014). Therefore, we propose using IL-2 along with zoledronic acid to improve 'effector lymphocyte' function *in vivo* in a NSG-tg(hu-IL15) mice model to revive SMG-induced loss of function.

Another hopeful strategy is to use granulocyte-colony stimulating factor (G-CSF) to counter spaceflight induced immune suppression. G-CSF is used to proliferate and mature white blood cells in neutropenia patients and hematopoietic stem cell donors. G-CSF has been shown to improve immunity against space radiation (M. Li et al., 2015). Therefore, combining G-CSF with other immunotherapeutic agents and timing it around heavy radiation exposure periods like passing through the Van Allen belts or instances of solar flares could prevent tumorigenesis in astronauts.

Limitations of spaceflight immunology literature:

- Post-flight data is confounded by landing G-stress. Therefore, it becomes complicated to discern effects of long-term spaceflight with late-mission time points in a 6-month mission. Some recent studies have collected samples just before return (R-1, one day before return) to circumvent this problem.
- In-flight samples are confounded by processing delays due to transfer to earth based laboratories.
- Different processing and testing methodology standards showing conflicting results between the US, International and Russian crew members.
- In-flight stress levels are not accurately represented by cortisol levels due to circadian misalignment. Cortisol levels in the body follow a diurnal pattern with highest levels in the morning. Altered circadian rhythm and sleep patterns might interfere with cortisol production. Cortisol:DHEA and ECS metabolite measurements have shown ambiguous and sometimes conflicting results.
- Radiation exposures beyond LEO might alter interactions with immune system. There is a lack of data in regards to space radiation effect on immune system. With the new GCR simulation equipment at the National space radiation laboratory (NSRL), there could be new data
- 20 times higher CO_2 levels on ISS (around 2.3-5.3 mm Hg) compared to Earth's 0.3 mm Hg has already been implicated in headaches, visual impairment due to intracranial pressure (VIIP) (Gibson, Alexander, & Hamilton, 2012). There is some evidence that altered CO_2 levels affect immune system (Cho et al., n.d.). Future studies could look into the systemic effect of high CO_2 levels on the immune system.

- Altered microbial virulence in confluence with reduced cell mediated immunity might compound the effect of immune dysregulation.
- Most countermeasures to immune dysregulation have been tested *in vitro* using SMG models. How these nutritional/therapeutic countermeasures would fare *in vivo* remains to be tested. This dissertation will involve testing the efficacy of a common *in vivo* countermeasure in a SMG mice model.
- Partial gravities of the Lunar and Mars surfaces are a novel stimulus to the human body. How this affects the immune system remains unknown.
- Lack of in vivo data is a major limitation to spaceflight data. Almost all immune studies involve *in vitro* testing. While it is unethical to perform human *in vivo* experiments concerning immune dysregulation and tumor development, mice models are beset with limitations of extrapolation to human physiology. This dissertation aims to find a middle ground using a humanized mice model with human immune cells and SMG.

Aims and Hypotheses

The planned longer duration spaceflights will result in greater exposure of astronauts to the deleterious effects of spaceflight, including immune dysregulation. We have previously shown that natural killer (NK) cell function is suppressed after 12-hour exposure to simulated microgravity (SMG) using a NASA-validated rotary cell culture system.

Specifically, when compared to NK cells that experienced 1G gravitational force, SMG-exposed NK cells kill 50% fewer cancer cells, have a reduced expression of cytotoxic granules

(perforin and granzyme B), and a reduced production of effector cytokines (TNFα and IFNγ) when exposed to cancer cells. Although *in vitro* models help to estimate the effect of short-term SMG on immune cells, there is a need to examine if these detrimental effects of SMG extend to more physiologically relevant *in vivo* models such as humanized murine models.

Additionally, clinical countermeasures for long duration exploratory class missions currently being considered have known immunological consequences. For example, zoledronic acid (ZOL), a bisphosphonate, may improve bone mineral density loss observed with spaceflight and subcutaneous IL2 may improve NK cell and γδ-T-cell function. Coincidentally, ZOL+IL2 is also used to improve *in vivo* anti-leukemia activity of γδ T-cell function and hasten immune reconstitution after a hematopoietic stem cell transplantation. Hence, it becomes pertinent to examine if ZOL+IL2 therapy, routinely used in a stem cell transplantation setting, can be used to boost *in vivo* anti-leukemia activity of human PBMCs after they have been exposed to SMG.

Problem statement

Therefore, there exists a need to examine the effect of SMG on both *in vitro* and *in vivo* function of various immune cells and evaluate the efficacy of common clinical therapies as potential countermeasures to SMG-induced effector lymphocyte loss-of-function.

Overarching hypothesis

We hypothesize that exposure to SMG adversely affects function of various immune cells both *in vitro* and *in vivo*; while systemic administration of 'ZOL+IL2' will boost the *in vivo* function of immune cells.

AIM 1:

Determine the effect of SMG on in vitro expansion and function of CMV-specific T-cells and γδ-T cells.

This aim will address the following questions:

Q1. Does SMG impair expansion of CMV-specific T-cells and/or γδ-T cells in vitro?

Q2. Does SMG impair function of CMV-specific T-cells and/or γδ-T cells in vitro?

via the following hypotheses:

Hypothesis 1a: Exposure to 12-hour SMG in a RCCS impairs in vitro expansion of CMV-specific T-cells and/or γδ-T cells.

Expected outcome: We expect fewer CMV-specific T-cells and/or γδ-T cells post-expansion after exposure to SMG.

Hypothesis 1b: Exposure to 12-hour SMG in a RCCS impairs in vitro function of CMV-specific T-cells and/or γδ-T cells.

Expected outcome: We expect reduced function of CMV-specific T-cells and/or γδ-T cells after exposure to SMG.

AIM 2:

Determine the effect of simulated microgravity on the in vivo anti-leukemia activity of human immune cells.

This aim will address the following question:

Q. Does SMG impair anti-leukemia activity of human immune cells in vivo?

via the following hypothesis:

Hypothesis 2: Exposure to 12-hour SMG in a RCCS adversely affects anti-leukemia activity of human immune cells in vivo by impairing their ability to control tumor growth in a NSG-tg(hu-IL15) mice model.

Expected outcome: We expect reduced anti-leukemia activity of human immune cells after exposure to SMG.

AIM 3:

Determine the effect of systemic administration of ZOL+IL-2 on the function of human immune cells in vivo and/or in vitro.

This aim will address the following question:

Q. Does systemic administration of ZOL+IL-2 boost the impaired anti-leukemia capacity of human immune cells in vivo and/or in vitro after exposure to SMG?

via the following hypothesis:

Hypothesis 3: Systemic administration of ZOL+IL-2 will boost the in vivo anti-leukemia capacity of human immune cells in an NSG-tg(hu-IL15) mice model and/or in an in vitro model.

Expected outcome: We expect that ZOL+IL-2 administration will boost the anti-leukemia capacity of human immune cells in an NSG-tg(hu-IL15) mice model.

Chapter 3

Material and Methods

1. Participants

Healthy adults between ages 18-49 who were willing to volunteer for the study were recruited and consent for blood draw was obtained according to Human Subjects Protection Program (HSSP) in compliance with the Institutional Review Boards (IRB) at University of Arizona and University of Houston.

2. Blood collection and processing

Blood was collected by a trained phlebotomist via venipuncture. Acid citrate dextrose (ACD) tubes or heparin tubes will be utilized for cell culture experiments, while serum-separating tubes (SST) were used to isolate serum to perform ELISAs for CMV status.

3. Rotary cell culture system

This culture system was originally designed by NASA at Johnson Space Center to culture tissues during spaceflight and was later licensed by Synthecon Inc., Houston, Texas USA. Rotary cell culture system (RCCS) was designed to suspend cells in the culture media to simulate the effect of microgravity. Simulation of microgravity is made possible in this system due to rotation of the high-aspect ratio vessels (HARV) on a horizontal axis. This rotation would facilitate the suspension of cells to coalesce into 3D aggregates and maintain a circular orbit within the vessel. This would result in the 3D aggregates to stay in continuous free fall while experiencing randomization of gravity vector (g) in a quiescent low-shear stress environment. Lymphocytes rotated at 10RPM will stay suspended within the vessel and

experience G-force equivalent to 10^{-2}G (RCCS manual, Synthecon Inc.). Two control vessels will be used. One vessel remains static allowing the immune cells to experience 1G gravitational force while the other vessel rotates on a vertical axis, which allows the cells to sediment to the bottom. The rotation of the media exposes the cells to shear stress, which will be used as rotational control. While one of the operating principles of RCCS was to expose the cells to a low-shear stress model (LSSM), this will account for any changes in function due to rotational shear stress while also experiencing 1G gravitational force, however minimal they might be. The experimental setup is shown in Figure 3.

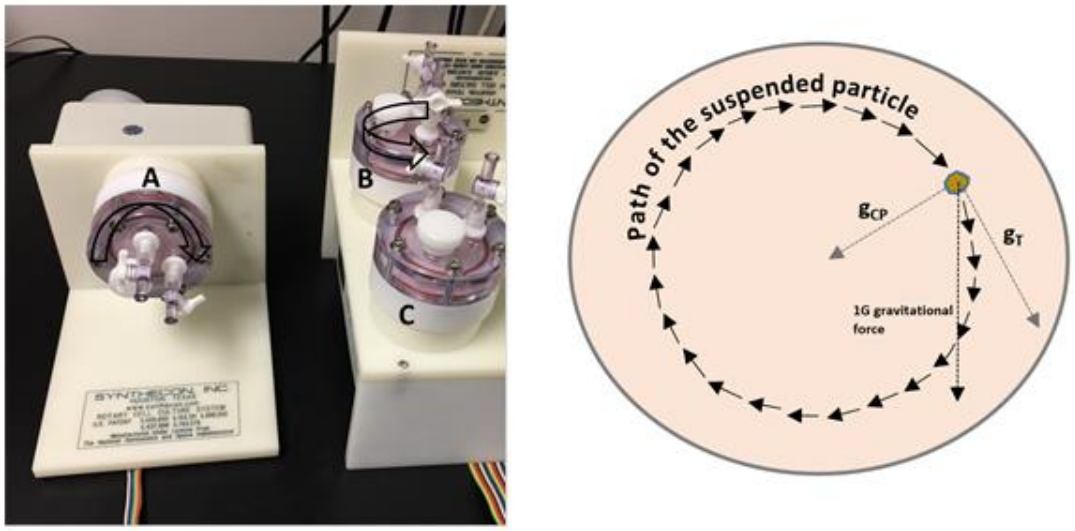


Figure 3. RCCS showing orientation of high-aspect ratio vessels (HARVs). Vessel 'A' rotates on a horizontal axis suspending cells in a circular orbit simulating microgravity (SMG). Vessel 'B' used as a rotational control (RC), rotates on a vertical axis in which the cells experience shear stress while also experiencing 1G gravitational force. Vessel 'C' remains static in which the cells experience only 1G gravitational force (STATIC) devoid of any shear stress. The suspended aggregate particle follows a circular path inside the vessel in a low-shear stress environment. The hydrostatic rotational force created by the rotating vessel and the gravitational (1G) force acting

on the particle will result in two partial 'g' vectors: g_{CP}, a centripetal force towards the center and g_T, a tangential force towards the momentum of traced particle.

4. Expansion of CMV-specific T-cells

Expansion protocols previously described (Gerdemann et al., 2009; Spielmann, Bollard, Kunz, Hanley, & Simpson, 2016a) using PBMCs from CMV+ subjects, CMV peptides (IE-1 and pp65) and cytokines IL-4, IL-7 and IL-15 (Miltenyi, Bergisch Gladbach, Germany) were used to expand CMV-specific T-cells in G-rex vessels (WILSONWOLF, Saint Paul, MN). Briefly, 10 million PBMCs from each condition were exposed to STATIC, rotational control and SMG conditions. Later, they were stimulated with CMV peptides, 400ng each of IE-1 and PP-65 (Miltenyi, Bergisch Gladbach, Germany), for one hour in an incubator (37C, 5% CO_2) in 50ml conical tubes. After stimulation, 15ml culture media containing 10% FBS+RPMI, 0.3µL/ml IL-4 (1666 I.U./ml) and 0.1µL/ml IL-7 (10ng/ml) per condition was added and transferred to G-rex vessels. After 5 days, media was replaced with 15ml 10% FBS+RPMI culture media that contains 0.3µL/ml IL-15 (5 ng/ml) in addition to 0.3µL/ml IL-4 (1666 I.U./ml) and 0.1µL/ml IL-7 (10 ng/ml) in each condition. Cell counts and phenotyping were performed at the end of expansion period using eight-color flow cytometry (MACS QUANT, Miltenyi, Bergisch Gladbach, Germany). CMV-specific T-cells were enumerated either with ELISPOT or with IFNγ secretion assay.

5. Expansion of γδ-T-cells

PBMCs from healthy human subjects were used to expand γδ-T cells using zoledronic acid along with 100 I.U./ml IL-2 as previously described (Kondo et al., 2011). Briefly, 10 million PBMCs from each condition were exposed to 1G (STATIC) control and SMG conditions. Later, they were plated in a flat bottom 24-well plate at a concentration of 1million cells per well in a culture media that contains zoledronic acid (5μM) and IL-2 (100 I.U./ml). The media was changed with culture media containing only IL-2 (100 I.U./ml) on days 3, 7, 10. Finally, the cells were harvested on day 14 for subsequent experiments. Cell counts and phenotyping were performed at the end of expansion period using eight-color flow cytometry (MACS QUANT, Miltenyi, Bergisch Gladbach, Germany).

6. Function of CMV-specific T cells

CMV-specific T-cell function was measured using a cytotoxicity assay against autologous PHA blasts (Spielmann et al., 2016a). CMV-specific T-cell functional assays included SMG exposed cells that were later expanded in 1G and conversely 1G expanded cells that were exposed to SMG at the end of expansion. Functional differences were compared within the three groups: STATIC, rotational control and SMG.

To evaluate the function of CMV-specific T-cells their cytotoxicity against autologous PHA blasts was estimated. PHA blasts were generated following manufacturer's instructions using phytohemagglutinin from Thermo Fisher Scientific (10576015). PHA blast generation protocol and cytotoxicity assay has been adopted from previous literature (LaVoy et al., 2015;

Spielmann et al., 2016a). 2% v/v PHA-M was used to stimulate 10million PBMCs in the presence of IL-2 (400IU/ml) for 72hours at 37°C. After blastogenesis, PHA blasts were cultured for 72 hours in culture medium (5% human serum+ RPMI). PHA blast cultures were unpulsed, pulsed with irrelevant peptide (MOG) or with CMV peptides (IE1, pp65) for 1-hour at 37°C. Following that, PHA blasts used as target cells were stained with CD3 (Viogreen) (Miltenyi Biotec, Bergisch Gladbach, Germany) to identify them. After two washes with PBS, target cells were incubated with CMV-specific T-cells at an effector: target cell ratio of 10:1. 0:1 effector: target cell ratio was used to estimate spontaneous cell death. Cryopreserved CMV-specific T-cells were thawed and incubated overnight in 100 IU/ml IL-2 at 37C 5% CO_2 before being used in the cytotoxicity assay. Specific lysis was used to estimate CMV-specific T-cell cytotoxicity. Data was presented as target cells lysed per 100,000 CMV-specific CD3+CD8+ T-cells.

7. Function of γδ-T-cells

γδ-T cell function was measured as specific lysis of cancer cell lines (K562 and U266) (Bigley, Spielmann, Agha, O'Connor, & Simpson, 2016). Briefly, target cells were initially stained with CD71 FITC (Miltenyi, Bergisch Gladbach, Germany). Later, γδ-T cells and target cells (K652 or U266) were co-incubated at different E:T ratios (0:1, 1:1, 5:1. 10:1 and 20:1) in u-bottom 96 well plates with 30,000 target cells in each well and in a final volume of 200µL in an incubator at 37C, 5% CO_2 for 4 hours. At the end of incubation, the cells were washed twice in PBS at 300G for 10 minutes. Cells were analyzed for dead target cells using flow cytometer immediately after adding propidium iodide (PI). PI+ target cells in 0:1 condition was

considered spontaneous cell death, while 'specific lysis'[1], a measure of γδ-T cell activity, was calculated for each E:T ratio. Data was presented at 10:1 Effector: Target cell ratio. 1G (STATIC) control and SMG conditions were compared within each experiment.

8. Gas-permeable rapid cell expansion vessels

Gas permeable rapid expansion (G-rex) vessels (WILSONWOLF, Saint Paul, MN) was used to expand CMV-specific T-cells by several folds (Bajgain et al., 2014). The gas permeable membrane in the bottom enables convectional exchange of O_2 from ambient air in the incubator into the vessel and escape of CO_2 from the media. This enabled us to perform phenotyping and functional assays (cytotoxicity, ELISPOT, and IFN- γ secretion assays).

9. CMV ELISA

CMV IgG qualitative ELISA kits from Immuno-biological laboratories, Inc. (IBL-America, Minneapolis, MN) was used to evaluate CMV status of the participants. Manufacturer's instructions were used to perform the ELISA and optical density of the wells was read using a luminescence plate reader at 450nm.

[1] Specific lysis = Total target cell death – spontaneous target cell death.

10. CMV ELISPOT

To enumerate the number of CMV-specific T-cells we used an enzyme linked immunospot assay (ELISPOT). ELISPOT for CMV specific T-cells was performed using the protocol previously mentioned (Spielmann, Bollard, Kunz, Hanley, & Simpson, 2016b). 10,000 cells per well from the expansion product were stimulated with CMV peptides: IE1 or pp65 for 12hours at 37 °C. Spot-forming cells (SFCs) were enumerated with Zellnet Consulting Inc. (Fort Lee, NJ, USA). Total SFCs were calculated and adjusted for number of CD3+ cells (% of cells from expansion product expressing CD3+). Data was presented as IE1 or pp65 reactive cells per 1million CD3+cells.

11. IFNγ secretion assay

To enumerate the number of CMV-specific T-cells we also used an IFNγ secretion assay. We followed manufacturer's instructions for the protocol obtained from Miltenyi Biotec (130-090-762). CMV-specific T-cells expanded in Aim 1a were reconstituted in culture medium (5% human serum+ RPMI) at a concentration of 10^7 cells/ml and stimulated with CMV peptides IE-1 and pp65 (10µg/ml) for 3 hours in an incubator at 37C, 5% CO_2. Later, they were washed in cold buffer (PBS at pH 7.2+ 0.5% bovine serum albumin+ 2mM EDTA). Followed by resuspension in cold culture medium, 15µL IFN-γ catch reagent was added and incubated on ice for 5 minutes. Followed by another incubation for 45 minutes at 37C after resuspension in 1mL warm culture medium. After two washes with cold buffer, 15µL IFN-γ detection antibody (APC) and 2µL each of CD8 (VioBlue), CD3 (VioGreen), CD4 (FITC), CD45 (APC-Vio770) was added and incubated for 15 minutes in the dark at 4C. After the incubation, and two washes

with PBS (300G, 10 minutes), the cells were analyzed on the flow cytometer. All antibodies were purchased from Miltenyi (Miltenyi Biotec, Bergisch Gladbach, Germany).

12. NSG-Tg(Hu-IL15) mouse model

All mouse experiments were done in compliance with Institutional Animal Care and Use Committee (IACUC) guidelines at the University of Arizona. Animals were purchased from Jackson laboratory (Bar Harbor, ME, USA) and breeding colonies were maintained at the Animal Care facility. Animals were housed at the University Animal Care facility.

Humanized NSG mice are severely immunodeficient mice that do not have a mature immune system (Leonard D. Shultz, Ishikawa, & Greiner, 2007). This makes them uniquely suited for *in vivo* models to study human hematopoietic system reconstitution, graft-versus-leukemia effect, graft-versus-host disease, in addition to studying chemotherapeutic drug toxicities, interactions, and efficacies in a pre-clinical stem cell transplantation setting.

Mutations in NSG-Tg(Hu-IL15) mice:

Prkdc^{scid} mutation in these mice cause 'severe combined immune-deficiency' resulting from absence of mature B and T cells (Bosma, Custer, & Bosma, 1983). **Il2rg^{tm1Wjl}** —a targeted mutation— in the common IL-2R gamma chain affecting IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 receptors prevents NK cell development, differentiation, and function along with reduced spontaneous generation of mature B and T cells (X. Cao et al., 1995; L. D. Shultz et al., 2005; Leonard D. Shultz et al., 2007). These mutations are on a NOD (non-obese diabetic) mouse

background that originally were intended to study autoimmune (type I) diabetes. These NOD mice inherently have several immune-deficiencies which include immature macrophages (Serreze, Gaedeke, & Leiter, 1993), defective antigen-presenting dendritic cells (Pearson et al., 2003), reduced NK cell activity (Kataoka et al., 1983) and a completely absent complement system (Nilsson & Muller-Eberhard, 1967).

Since all these immune-deficiencies congregate in NSG mice, the mice do not have a capable immune system to mount a resistance to xenogeneic transplantation with human CD34+ (hematopoietic stem cells) cells or peripheral blood stem cell (PBSC) grafts resulting in improved engraftments (L. D. Shultz et al., 2005).

NK cells develop in the bone marrow microenvironment and need IL-15 for their development (Fehniger & Caligiuri, 2001; Williams et al., 1998). *In vivo* survival of NK cells is also predicated on the exogenous supply or indigenous production of IL-15 (Cooper et al., 2002). Therefore, when a transgenic insertion of humanized IL-15 sequence (**Tg(IL15)1Sz/SzJ**) is added onto the NSG mouse background, this resulted in enhanced development and survival of human NK cells from the stem cell graft in the NSG mice (Brehm et al., 2018).

Since we propose to study the effect SMG on *in vivo* anti-leukemic activity of human PBMCs, which is largely driven by NK cells, we will utilize this NSG-Tg(Hu-IL15) mice model which enhances *in vivo* survival of human NK cells. Our experiments suggested that acute GVHD starts occurring around 14 days after PBMC injection and severe GVHD occurs around day

28. Therefore, we chose to evaluate graft-versus-tumor effect before severe GVHD starts setting in to prevent GVHD associated immune modulation from eclipsing the graft-versus-tumor effect of human PBMCs. Acute GVHD scoring will be based on previously published grading (Lai, Chou, Tzeng, & Lee, 2012; Naserian et al., 2018).

GVHD scoring: GVHD will be scored on 6 parameters:

1. Activity: normal=0, mild to moderately inactive=1, severely inactive=2.
2. Diarrhea: absent=0, present=1.
3. Fur: normal texture=0, mild to moderate ruffling=1, severe ruffling=2.
4. Posture: normal=0, hunching at rest=1, severely hunching=2.
5. Skin integrity: normal=0, scaling of paws and tail=1, obviously denuded=2.
6. Weight loss: 0-9.99%=0, 10-19.99%=1, ≥20%= sacrifice criteria met.

An acute GVHD score of 4 or more will be considered termination point of the experimental data collection. NSG-Tg(Hu-IL15) mice will be obtained from Jackson labs and breeding colonies will be maintained at the University of Arizona Animal Care Facility.

Zoledronic acid+IL-2 therapy:

Administration of zoledronic acid and interleukin-2 has been shown to improve *in vivo* γδ T cell activity against various tumors in both human trials and murine models (Ottewell et al., 2008; Pressey, Adams, Kelly, & Lamb, 2013). Low doses of subcutaneous IL-2 has shown to

expand NK cells *in vivo* (Meropol et al., 1996), improve function of NK-cells, γδ-T-cells, T-cells and B-cells (Gaffen & Liu, 2004; Mahmoudpour et al., 2019). Therefore, we propose to test the efficacy of 'ZOL+IL-2' therapy as a potential countermeasure to SMG induced loss-of-function *in vivo.* All mouse care and experimental procedures were performed in accordance with the guidelines of Institutional Animal Care and Use Committee (IACUC) at the animal care facility at the University of Arizona.

13. Bioluminescent imaging

K-562-luc2 (strain: ATCC® CCL-243-LUC2™) (American Type Culture Collection, Manassas, VA, USA), which is a K562 (chronic myeloid leukemia) cell line that has been tagged with luciferase will be used for *in vivo* experiments. When cells bearing luciferase encounter luciferin, catalytic oxidation of luciferin will result in emission of photons. This combined with negligible auto fluorescence of regular cells and tissues will result in high signal-to-noise ratio which can be used for *in vivo* imaging applications (Duda, Karimi, Negrin, & Contag, 2007; Paley & Prescher, 2014). After K-562-luc2 has engrafted in the mice, tumor burden can be measured when D-luciferin (GoldBio, St Louis, MO) is injected intraperitoneally. A LagoX spectral imager (Spectral Instruments Imaging, Tucson, AZ) will be used to obtain bioluminescent intensity (BLI) scores.

14. Statistical analysis

Statistical analysis was performed on Graphpad Prism 8.4.3 software (Graphpad, San Diego, CA). Statistical significance level was set at $p<0.05$.

CMV-specific T-cell expansion numbers and IFNγ secretion assay results met normality (Shapiro-Wilk test), equality of variances (Brown-Forsythe test), and normality of residuals assumptions. Therefore, a one-way RM ANOVA was used to analyze the data without assuming sphericity and Geisser-Greenhouse correction was applied. PHA blast cytotoxicity data for CMV-specific T-cells was analyzed using RM ANOVA (for day 14) or Friedman test (for day 15) in compliance with normality assumption. CMV ELISPOT enumeration data met normality assumption (Shapiro-Wilk test). So, a paired t-test was used to analyze the data since there were only two groups (1G control and SMG).

Γδ-T cell expansion numbers failed normality test. Therefore, a two-tailed Wilcoxon matched-pairs signed rank test was performed to evaluate differences between the groups. Γδ-T cell phenotyping: paired t-test was used when normality assumption was met. When normality test failed, Wilcoxon matched-pairs signed rank test was used. Day14 Γδ-T cell numbers, day 14 and 15 vγ9vδ2 and vγ9vδ1 proportions, day 15 NKG2D+ proportions, day 14 and day 15 NKp30+ proportions, day 14 CD3- CD56+, day 15 NKG2D+ CD158b- proportions failed normality tests. All Γδ-T cell cytotoxicity data passed normality tests, so a two-tailed paired t-test was used since there were two groups (1G control and SMG).

For AIMS 2 and 3, for tumor progression bioluminescent intensity data was log-transformed for normality as previously done for BLI data (Stokes, Hoffman, Zeng, Larmonier, & Katsanis, 2016). A mixed effects model was used to analyze differences between tumor growth with 'condition', 'time' main effects and 'condition*time' interaction effect in the model. When 'condition*time' interaction effect was significant in the whole model (suggesting that tumor growth varied between conditions as the experiment progressed), systematic pairwise comparisons were conducted to identify which conditions were significantly different from each other as time progressed. Peak BLI was analyzed using Friedman test for paired comparisons. For GVHD scores and engraftment data were analyzed using a linear mixed model or a two-way RM ANOVA with main effects of condition and time as well as an interaction term condition*time was added into the model. A log-rank (Mantel-Cox) test was used for survival analysis.

15. Rationale for 12-hour SMG exposure

Exposure to SMG was limited to 12 hours. This ensured optimal viability of PBMCs after exposure to SMG, enabling functional analysis without viability being a confounding factor. While various groups have used extended exposure to SMG with the help of cytokines that extend viability ex vivo, this will invariably affect functionality. This could mask any effect of a novel stimulus like SMG on uninitiated effector immune cells.

Piloting experiments in our lab showed that while human lymphocytes retained >90% viability up to 12 hours in SMG. Extending exposure to SMG beyond 12-hours increased the proportion

of lymphocytes entering early apoptosis—identified by annexin V+ve staining—indicating decrements in viability. Extending SMG exposure beyond 12-hours also increased proportion of lymphocytes entering late apoptosis/necrotic stage—identified by annexin V +ve and propidium iodide +ve— indicating increased plasma membrane permeability, a sign of cell entering the apoptotic cycle. Figure 4 shows representative flow cytometry plots of 1G control and SMG exposed lymphocytes after various (0, 6, 12 and 18 hours) time-periods along with viability graphs. Viable lymphocytes were identified within the forward and side scatter of lymphocyte gate within the PBMC sample and later gated on CD45+ve to exclude any large debris. Viability at zero hour was 98.4±0.4 (Mean±SE). After 6-hours, viability was stable in both 1G-control (97.4±0.27) and SMG (96.9±0.42) conditions and at 12-hours; viability was still comparable between 1G-control (93.4±0.63) and SMG (91.6±0.98). When exposure to SMG was extended to 18-hours, the viability dropped to 85.87±2.45 while 1G-control retained viability above 90% at 90.34±1.23. Therefore, we restricted exposure to SMG for 12-hours.

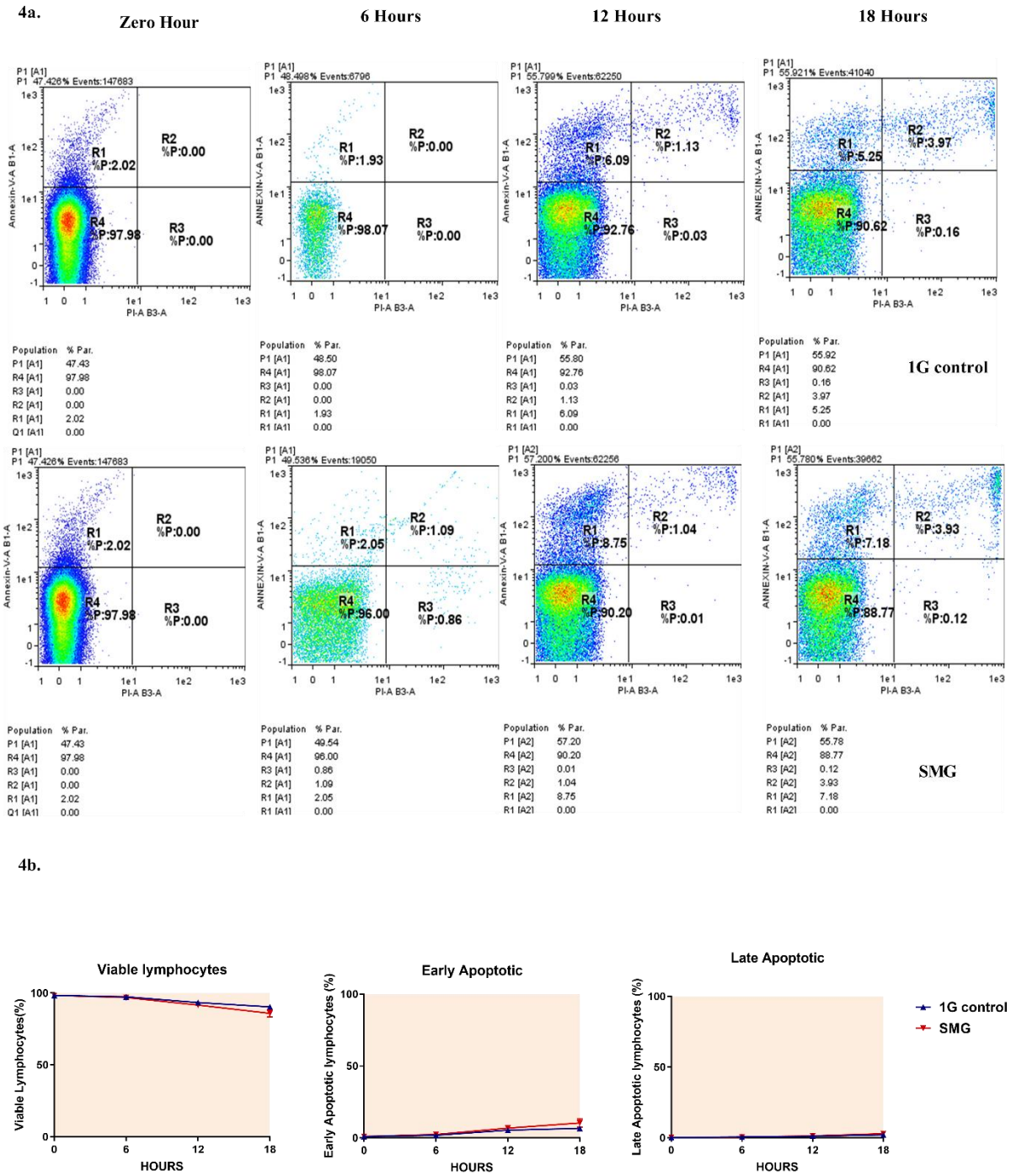


Figure 4 4a. Representative flow cytometry plots showing lymphocytes with annexin V/PI staining after exposure to 1G control (top row) and SMG (bottom row). Lower left quadrant of the plot shows viable cell population (annexin V –ve and PI -ve), upper left quadrant shows early

apoptotic cells (annexin V +ve and PI -ve), upper right quadrant shows late apoptotic cells (annexin V +ve and PI +ve). Figure 4b. shows average proportions of viable (left), early apoptotic (center) and late apoptotic (right) lymphocytes after 0, 6, 12 and 18 hours of exposure to 1G control (blue line) and SMG (red line).

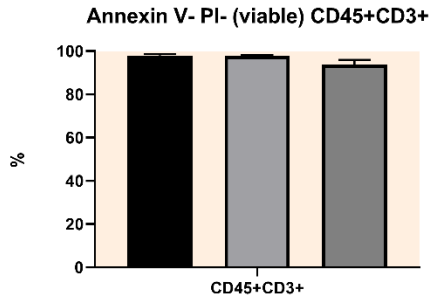
16. Preservation of PBMC subsets and NK cell proportions after 12-hour SMG exposure

Proportions of major lymphocyte subsets were not altered due to differential attrition from exposure to SMG. Especially, the effector lymphocytes of the innate immune system crucial to MHC-unrestricted killing (NK cells) were not depleted in the population. T-cells appear to be more susceptible to entering apoptotic phase due to SMG exposure with CD3+CD4-CD8- (presumptive γδ-T cells) showing 9.84±4.57% Annexin V+ staining and CD3+CD4+ T-cells showing 2.42±1.32% entering late apoptotic phase (figure 5e. and 5f.). Viable NK cells (figure.5d.) after exposure to SMG (98.46±0.67, MEAN±SE) remained at levels comparable to both STATIC-1G (99.21±0.28) and rotational-1G (99.33±0.22) controls. While more NK cells entered early (1.1±0.44) and late apoptotic (0.16±0.11) stages in SMG condition compared to both 1G controls, the levels 1.1% and 0.16% respectively (figures 5g. and 5h.), are not significant enough to account for functional differences observed between the conditions in both in vitro and in vivo experiments. Furthermore, NK cells that have been exposed to SMG successfully engrafted and have undergone an in vivo expansion in NSG-tg(hu-IL15) mice corroborating their viability.

Limitations of 12-hour SMG exposure

- Short-term exposure might only reveal initial effects of SMG exposure.
- Limited extrapolation to long-term effect of SMG *in vivo*.
- Effects of microgravity on a systemic level might be obscured.

5a.



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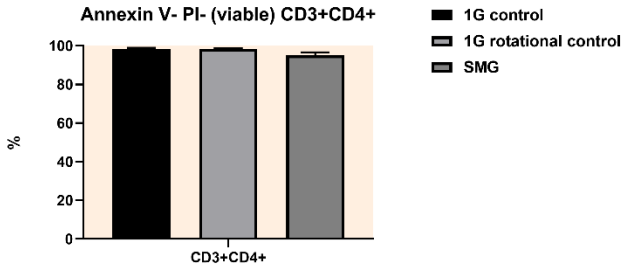

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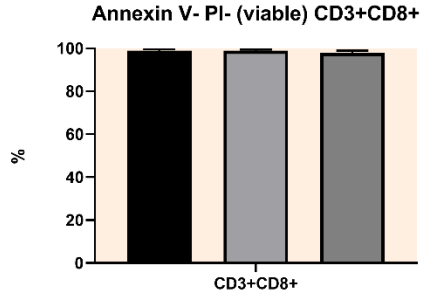

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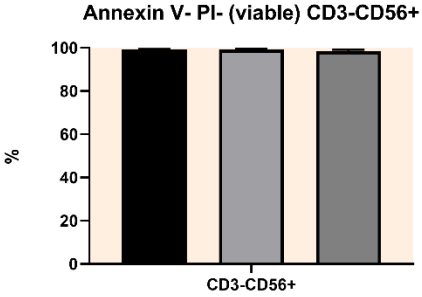

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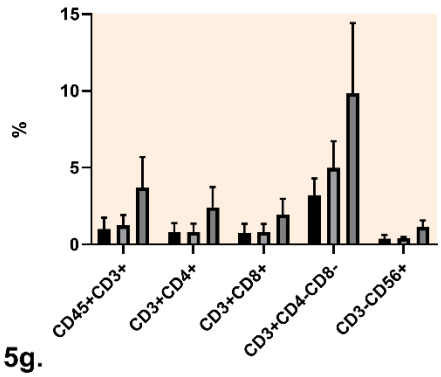

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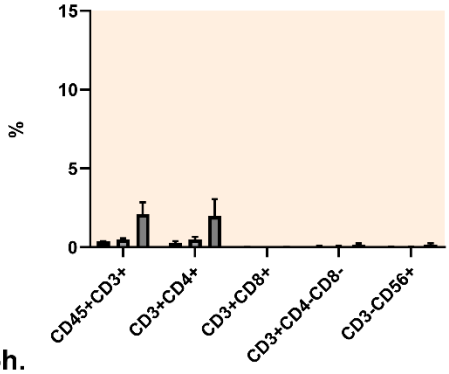
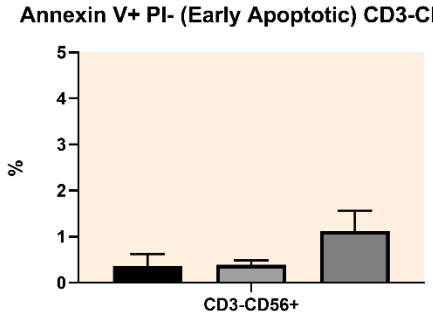


5g.



5h.

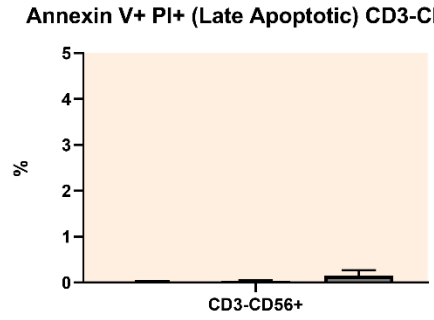


Figure 5. Viable total T-cells (4a.), T-helper cells (4b.), cytotoxic T-cells (4c.) and natural killer cells (4d.) were comparable in both 1G controls (static and rotational control) with SMG exposed lymphocytes. Less than 5% total T-cells entered early apoptosis and 2% of total T-cells entered late apoptosis (4e. and 4f.). 1.1% and 0.16% of NK cells have entered early and late apoptosis respectively (4g. and 4h.) N=4, MEAN±SEM.

Chapter 4

Effect of Simulated microgravity on expansion potential and function of γδ-T cells and CMV-specific T-cells.

1. Introduction

Latent viral reactivation is a consistently observed phenomenon during spaceflight. 53% of astronauts on shuttle missions and 61% of ISS crew members have shown shedding of at least one latent herpes virus (Rooney et al., 2019). 61% of the ISS crew shed CMV at detectable levels during the mission. CMV seroprevalence in the United States is around 50% (Bate, Dollard, & Cannon, 2010). CMV reactivation symptoms include CMV retinitis (Port et al., 2017), pneumonitis (Balthesen, Messerle, & Reddehase, 1993) and ulcerative colitis (Sager, Alam, Bond, Chinnappan, & Probert, 2015) in immune-compromised individuals. While CMV causes significant comorbidity in immune-compromised individuals, it does not seem to clinically burden immune-competent individuals (Cook, 2007).

Therapeutic interventions for CMV reactivation might be ineffective at reducing morbidities in critically ill immune-competent individuals (Heininger et al., 2001; Jaber et al., 2005). This is true in terrestrial settings. However, immune dysregulation observed during spaceflight combined with CMV reactivation might further incapacitate or reduce efficiency of crewmembers during long-duration missions. Severe CMV reactivation and its associated clinical symptoms could be a major medical emergency during an exploratory class mission with limited exploration medical capabilities (B. Crucian, Kunz, & Sams, n.d.).

Control of latency of CMV infection is achieved when there is a balance between viral load and CMV-specific T-cells (van der Heiden, Marijt, Falkenburg, & Jedema, 2018). Successful control of CMV infection has been correlated with CD4+ and CD8+ CMV-specific T-cells in

the memory compartment (Sylwester et al., 2005). Maintenance of the CMV-specific repertoire in the CD8+ effector memory compartment also is needed to control CMV latency (Klarenbeek et al., 2012).

Older populations show a dominance of CMV-specific T-cells in the CD8+ T-cell compartment underscoring the impact latent CMV infection has on the immune system (Hosie et al., 2017). Average age of astronaut population is 44.5 years for men and 42.5 years for women (Goel et al., 2014) making it pertinent to examine the effects of spaceflight on CMV-specific T-cell compartment. Additionally, NK cell function is also important to maintain latency and control CMV reactivation (Biron, Byron, & Sullivan, 1989; Polić et al., 1998). We have previously shown that NK cell function might be impaired in astronauts (Bigley et al., 2018).

These concerns make CMV-specific T-cell function crucial to maintaining immune health during long-duration exploration class missions. However, there is lack of data on how microgravity affects CMV-specific T-cell expansion potential and function.

Γδ-T cells possess γ and δ chains in their heterodimeric T-cell receptor, distinguishing them from the conventional T-cells that possess α and β chains. Γδ- T cells account for 0.5-5% of total T lymphocytes (Zhao, Niu, & Cui, 2018). This makes them a much smaller subpopulation of the lymphocytes compared to NK cells, which boast a strong contingent of 5-15% of all lymphocytes. However, their role in tumor transformation, especially in the early stages, is

being recognized owing to their tissue heterogeneity and multiple alternative pathways to activation (Pauza et al., 2018).

While they can be activated through the conventional TCR-peptide-MHC interaction, they also possess natural killer receptors (NKRs) including activating receptors NKG2D, NKG2C, DNAM-1, NKp30 and NKp44 and killer inhibitory receptors (KIRs) like NKG2A, KIR2DL3 etc. (Correia, Lopes, & Silva-Santos, 2013). The unconventional TCR's ability to recognize stressed cells via pathogen associated molecular proteins (PAMPs) and the innate-like ability to recognize "stressed cells" without relying on MHC molecules (like NK cells) make them a cell population that possess features of both innate and adaptive immune system.

Two major subsets of γδ-T cells: Vδ1 and Vδ2, exhibit high degree of tissue heterogeneity. Vδ1 γδ-T cells are mostly restricted to intra-epithelial layers, where they recognize various tissue stress signals like heat shock proteins and viral proteins. This makes them important to maintain epithelial health (Nielsen, Witherden, & Havran, 2017). Vδ2 γδ-T cells are the dominant subset in the peripheral blood compartment, with ability to recognize phosphoantigens. Phosphoantigens are a major by-product of mevalonate pathway, which is considerably upregulated after malignant transformation. This makes the circulating Vδ2 γδ-T cells ideal to control leukemias (Pistoia et al., 2018). Subsets of γδ-T cells that possess immunosuppressive properties that might promote tumor progression in tumor microenvironment have also been studied (Fleming, Morrissey, Cai, & Yan, 2017). Their possession of a broad cytokine profile can also be used to compound immune responses (Lawand, Déchanet-Merville, & Dieu-Nosjean, 2017). All these features make γδ-T cells an important subset in the immune system. However, the effect of microgravity on these cells has

not been explored. In this dissertation, effect of simulated microgravity on CMV-specific T-cells and γδ-T cells has been explored.

2. Experimental Design

Hypothesis 1a[2]:

To evaluate the effect of exposure to SMG on CMV-specific T-cell expansion: PBMCs from CMV+ve subjects—identified with CMV ELISA— were exposed to 1G-control or SMG and later exposed to CMV peptides and expanded using the protocol detailed earlier. The expansion potential of CMV-specific T-cells from being a tiny portion of the T-cells to functionally significant proportion of the effector-memory compartment would dictate how well a latent viral reactivation—like those seen in astronauts on an ISS mission—could be curtailed from becoming a clinical manifestation. Hence, it is pertinent to examine if a modeled short-term exposure to SMG might impair the expansion potential of CMV-specific cells. Using a paired-sampling in vitro expansion model (Figure 6) controlled for any differences in subsets and the maturation stages of the subsets. The culture media was devoid of IL-15 until the first media change (day5) preventing growth of unwanted PBMC subsets; especially NK cells. This is to prevent NK-cell contamination from over-estimating killing capacity in functional assays. CMV-specific T-cells were enumerated using either ELISPOT or flow cytometry using IFNγ secretion assay.

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[2] "Exposure to 12-hour SMG in a RCCS impairs in vitro expansion of CMV-specific T-cells and/or γδ-T cells."

Enumeration of CMV-specific T-cells:

CMV-specific T-cells were enumerated with an ELISPOT or using the IFNγ secretion assay. ELISPOT was used to enumerate CMV-specific T-cells from total VST expansion product as well as to test the ability of immune cells exposed to SMG to recognize their cognate antigen. This was done *a priori* to performing secretion and functional assays to prevent structural inability of CMV-specific T-cells to recognize their antigen from confounding later analyses. The data is presented as CMV-specific T-cells per million T-cells. IFNγ secretion assay gating strategy is detailed in Figure 7. CD8 (Vioblue), CD3 (Viogreen), CD4 (FITC), propidium iodide (PerCP), IFNγ (APC), CD45 (APC Vio770) were used in the phenotyping panel for the IFNγ secretion assay.

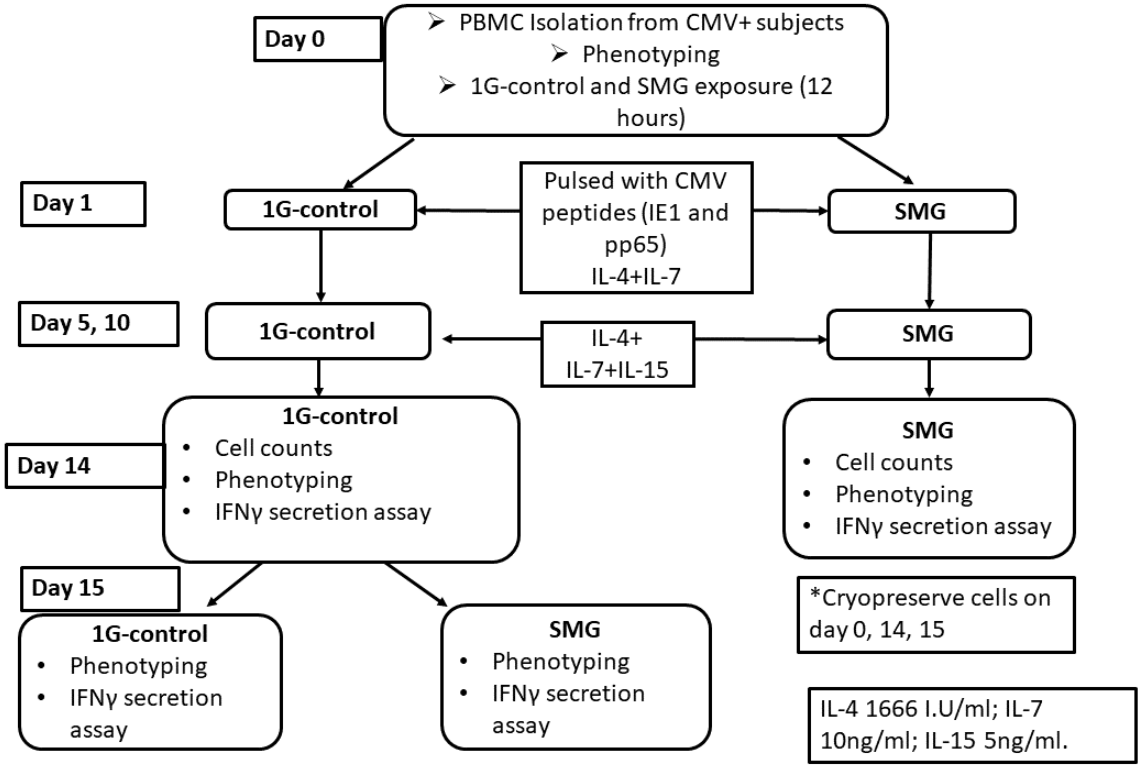
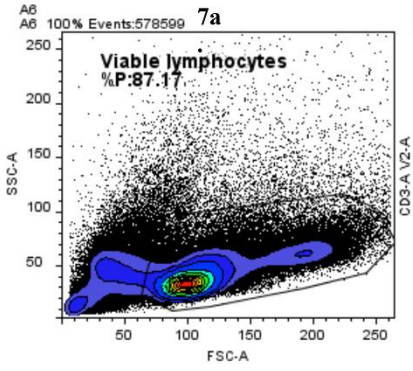
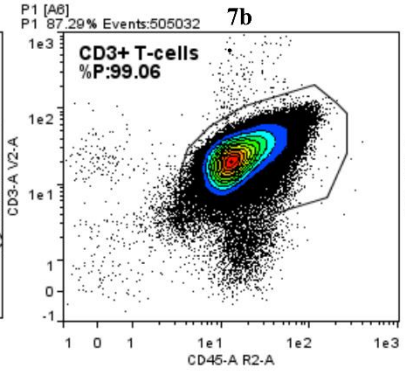


Figure 6. Experimental study design to evaluate the effect of SMG on CMV-specific T-cells.



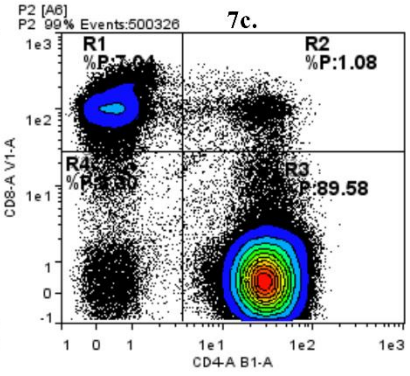
7a

Population	% Par.
A6	100.00
Viable lymphocytes [A6]	87.17



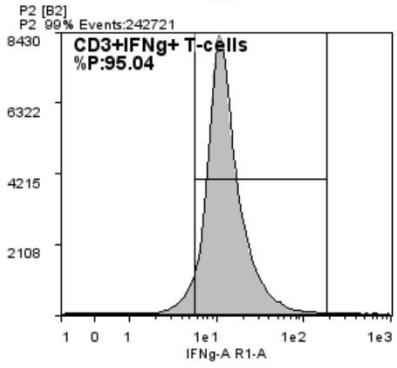
7b

Population	% Par.
P1 [A6]	87.29
P2 [A6]	99.06



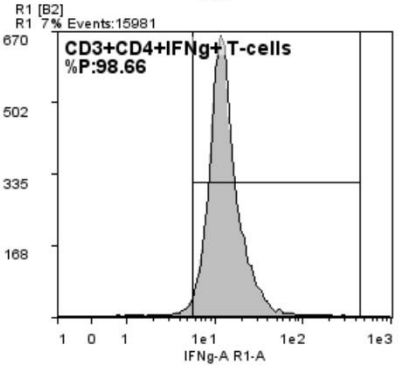
7c.

Population	% Par.
P2 [A6]	99.14
R4 [A6]	2.30
R3 [A6]	89.58
R2 [A6]	1.08
R1 [A6]	7.04
Q1 [A6]	0.00
M1 [A6]	0.41



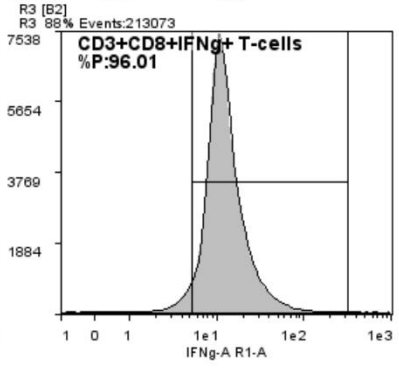
7d.

Population	% Par.
R4 [B2]	4.42
R3 [B2]	87.79
R2 [B2]	1.21
R1 [B2]	6.58
R1 [B2]	0.00
CD3+IFNg+ T-cells [B2]	95.04



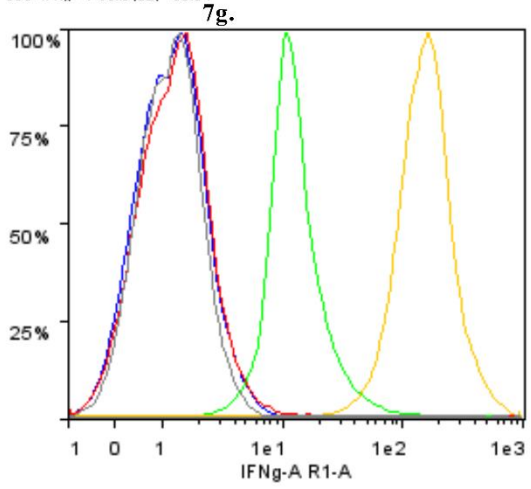
7e.

Population	% Par.
R1 [B2]	6.58
CD3+CD4+IFNg+ T-cells [B2]	98.66

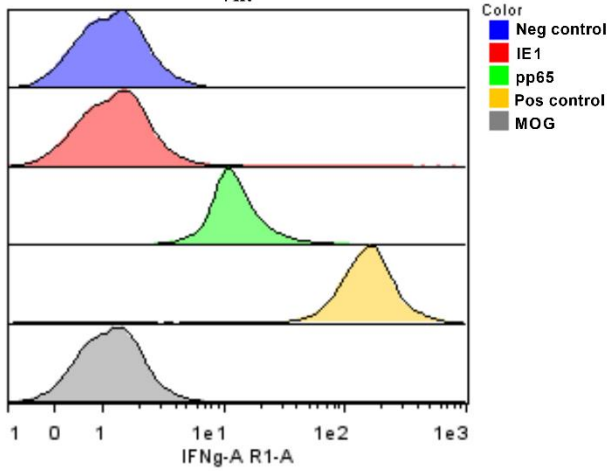


7f.

Population	% Par.
R3 [B2]	87.79
CD3+CD8+IFNg+ T-cells [B2]	96.01



7g.



7h.

Figure 7. Interferon-gamma secretion assay. 7a. Identification of viable lymphocytes. 7b. Identification of CD3+ T-cells. 10c. Identifying CD4+ and CD8+ T-cells. 7d-f. Identifying IFNγ+ cells in total CD3+ and CD4+, CD8+ subsets. 7g-h. Histogram overlays showing negative control, positive control and irrelevant peptide (MOG) stimulation control.

To evaluate the effect of exposure to SMG on γδ-T cell expansion: PBMCs from healthy human subjects were exposed to 1G-control or SMG for 12-hours and later expanded using previously described ex vivo expansion protocol (Kondo et al., 2011) (Figure 8). At the end of the 14-day expansion, the number of γδ-T cells were enumerated on the flow cytometer. Phenotypic differences for major subsets of γδ-T cells (vγ9 vδ2 and vγ9 vδ1) along with differences in major activating and inhibitory receptors were measured on flow cytometer using the phenotyping panel in Table 1. Detailed expansion protocol is mentioned earlier.

Table 1. γδ-T cell phenotyping panel.

	Vioblue	Viogreen	FITC	PE	Per-CP	PE-Vio770	APC	APC-Vio770
Panel 1	CD8	CD3	vγ9 vδ2	CD4	CD45	-	vγ9 vδ1	CD56
Panel 2	CD16	CD3	-	NKG2D	PD-1	CD158b	NKG2A	TCR-γδ
Panel 3	-	CD3	-	NKp30	-	-	NKG2A	TCR-γδ

Hypothesis 1b[3]:

To evaluate the immediate effect of exposure to SMG on CMV-specific T-cell function: cells that were expanded in 1G and were later exposed to SMG at the end of expansion were used in a 4-hour PHA blast cytotoxicity assay detailed earlier. Conversely, to evaluate the residual effects of exposure to SMG on CMV-specific T-cells, much akin to the design mentioned in γδ-T cell experiments, CMV-specific T-cells that were exposed to SMG and later expanded in 1G were stimulated with CMV peptides and their cytotoxicity against autologous PHA blasts was measured.

To evaluate the immediate effect of exposure to SMG on cytotoxicity of γδ-T cells: cells that were expanded in 1G and were later exposed to SMG at the end of the 14-day expansion, were co-incubated with chronic myeloid leukemia (K562) and multiple myeloma (U266) cell lines in a 4-hour killing assay. Conversely, to evaluate the residual effects of exposure to SMG on γδ-T cell function: γδ-T cells that were exposed to SMG and later expanded in 1G for 14-days were co-incubated with K562 and U266 in a 4-hour killing assay detailed earlier. The latter would identify any long-term residual effects of exposure to SMG, since the 14-day expansion in 1G would accommodate for any reparative cellular processes. Therefore, functional differences would be representative of any residual detrimental effects of SMG that were sustained after the 14-day expansion.

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[3] "Exposure to 12-hour SMG in a RCCS impairs in vitro function of CMV-specific T-cells and/or γδ-T cells."

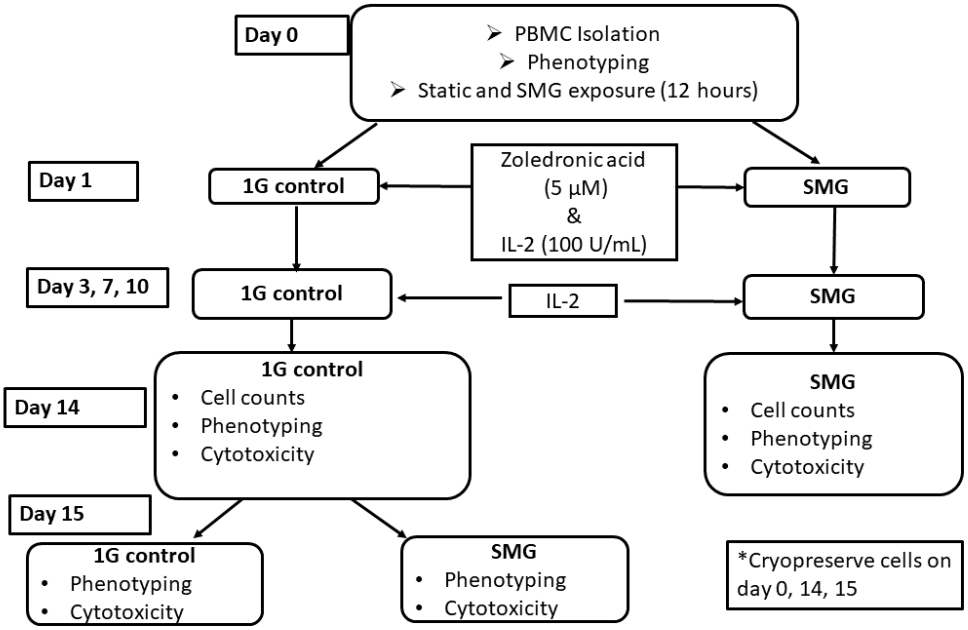


Figure 8. Experimental study design to evaluate the effect of SMG on γδ-T cells.

3. Results

Hypothesis 1a:

3a. Exposure to 12-hour SMG in a RCCS impaired in vitro expansion of CMV-specific T-cells

Exposure to SMG impaired the ability of CMV-specific T-cells to expand compared to both 1G-exposed controls (RM ANOVA, $F(1.571, 6.283)= 8.367$, $p=0.0198$). 10-million PBMCs at day1 of the expansion in SMG-exposed condition yielded 17.63±3.75 million (MEAN±SEM, N=5) CMV-specific T-cells at the end of the expansion. In comparison, STATIC-1G control cells expanded to 33.8±7.57 million, while 1G-rotational control exposed cells expanded to 28.32±7.21 million cells (Figure 9).

ELISPOT enumeration of the number of CMV-specific T-cells per million T-cells using ELISPOT also showed that SMG-exposure did not impair their ability to later expand and detect antigens compared to 1G-control (paired t test, $t(3)=1.543$, $p=0.22$). SMG exposed PBMCs contained 4654±2397 (N=4, MEAN±SEM) CMV-peptide detecting VSTs per 1 million CD3+ T-cells while 1G-control PBMCs contained 1062±465 CMV-peptide detecting VSTs (Figure 10).

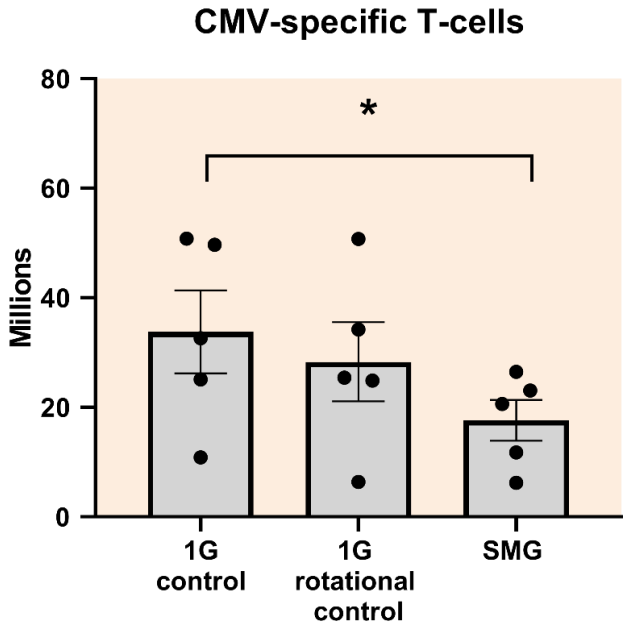


Figure 9. Exposure to SMG suppressed CMV-specific T-cell ability to expand in a 14-day expansion. N=5, MEAN±SEM, RM ANOVA, p=0.0198.

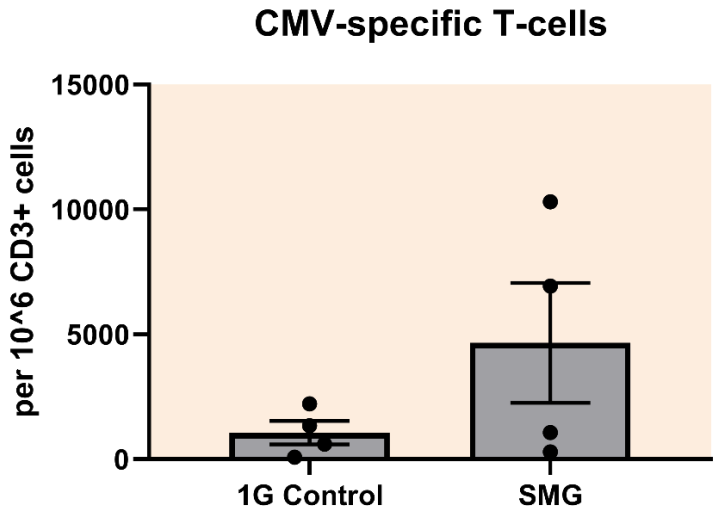


Figure 10. Exposure to SMG did not hinder CMV-specific T-cells' ability to detect CMV peptides later. N=4, MEAN±SEM, paired t test, p=0.22.

3b. Exposure to 12-hour SMG in a RCCS impaired in vitro expansion of γδ-T cells.

Exposure to SMG impaired the ability of γδ-T cells to expand compared to 1G-control (Wilcoxon signed ranks test, p=0.039). 10 million PBMCs at day 1 of the expansion in SMG-exposed condition yielded 102.3±23.07 million (MEAN±SEM, N=9) γδ-T cells at the end of the expansion. In comparison, 1G-control PBMCs yielded 113.7±23.91 million γδ-T cells (Figure 11).

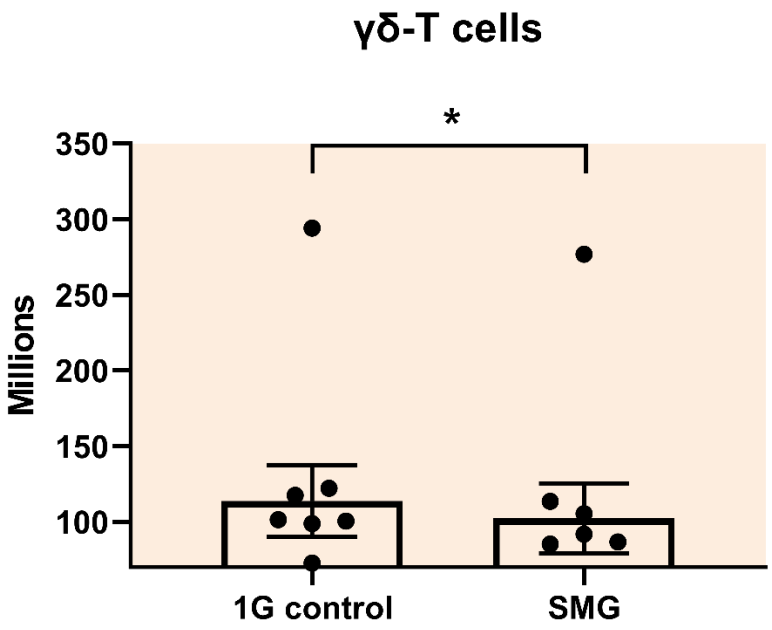


Figure 11. Exposure to SMG suppressed naïve γδ-T cell ability to expand in a 14-day expansion. N=9, MEAN±SEM, Wilcoxon matched-pairs signed rank test, p =0.039.

Hypothesis 1b:

3c. Exposure to 12-hour SMG in a RCCS did not impair in vitro function of CMV-specific T-cells.

In vitro function of CMV-specific T-cells was measured as cytotoxicity against autologous PHA blasts generated from the donor's PBMCs (Figure 12).

Exposure to SMG did not impair CMV-specific T-cell ability to kill target cells compared to 1G and 1G-rotational control (RM ANOVA, $F(1.357,12.21)=0.7434$, $p=0.4457$). 100,000 cytotoxic (CD8+) CMV-specific T-cells exposed to SMG at the end of expansion killed 804.6±166.3 (MEAN±SEM, N=10) autologous PHA blasts pulsed with CMV peptides. In comparison, 1G-control and 1G-rotational control killed 909.2±160.6 and 669.7±125 PHA blasts respectively (fig. 12d).

To examine the residual effects of exposure to SMG on CMV-specific T-cell function, PBMCs were exposed to SMG and CMV-specific T-cells were later expanded in 1G. Cytotoxic activity was measured in the expanded product. Exposure to SMG did not impair CMV-specific T-cell ability to later expand and kill target cells in 1G (RM ANOVA, $F(1.976,17.78)=3.875$, $p=0.04$). While the overall model was significant, post-hoc analysis using Tukey's multiple comparisons test, showed SMG exposed CMV-specific T-cells that were later expanded in 1G killed more target cells compared to 1G-rotational control ($p=0.039$) but not 1G-control ($p=0.36$). 100,000 cytotoxic (CD8+) CMV-specific T-cells exposed to SMG and later

expanded in 1G killed 948.3±154.1 (MEAN±SEM, N=10) autologous PHA blasts pulsed with CMV peptides. In comparison, 1G-control and 1G-rotational control killed 770.5±152.8 and 616.5±135.1 PHA blasts respectively (fig. 12c).

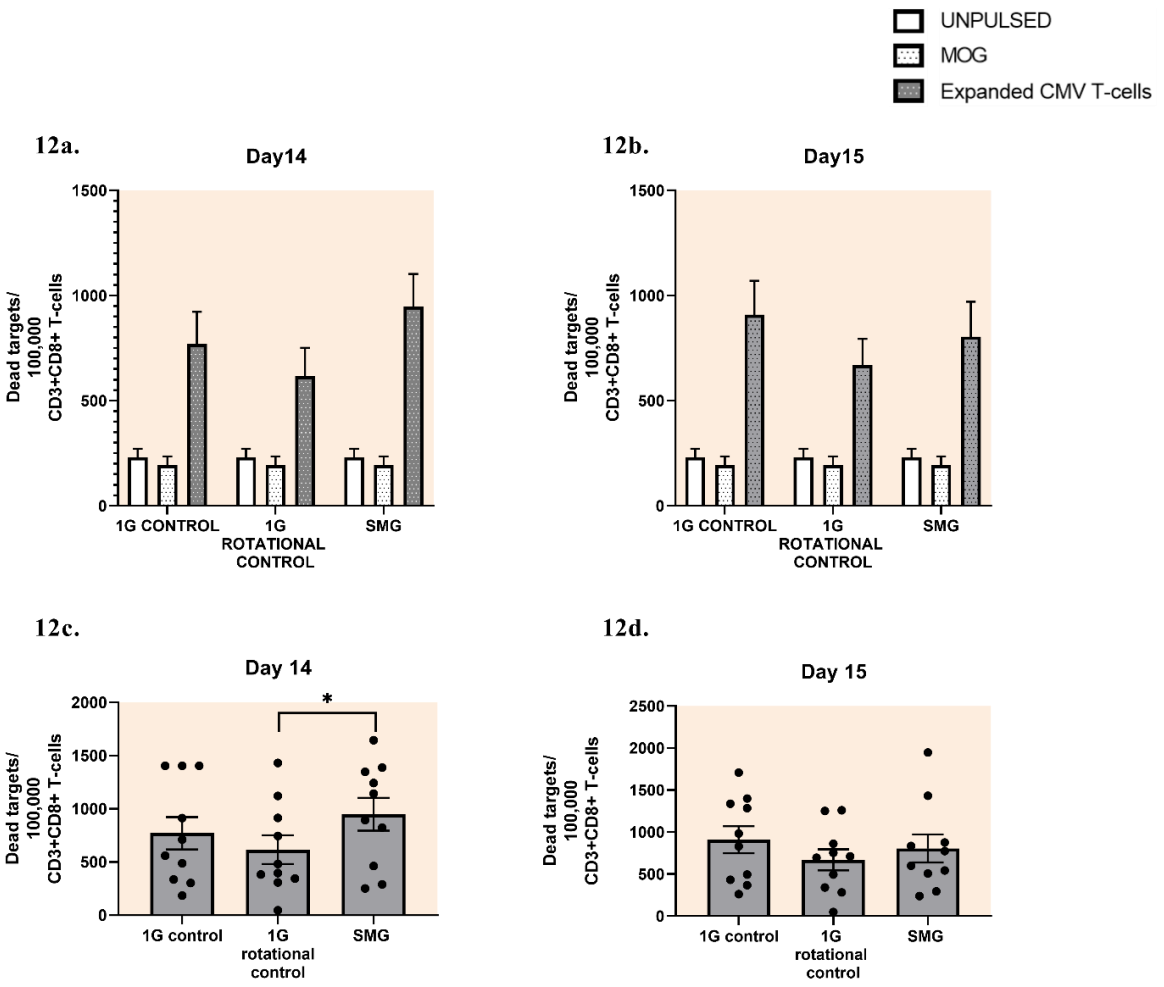


Figure 12. CMV-specific T-cell cytotoxicity assay. 12a-b. shows CMV-specific T-cells' cytotoxicity against autologous PHA blasts when left unpulsed, pulsed with irrelevant peptide (MOG), or pulsed with CMV peptides (IE1+pp65). 12c. shows CMV-specific T-cells' cytotoxicity after being exposed to SMG and later expanded in 1G. SMG>1G-rotational control, p=0.039,

N=10, MEAN±SEM. 12d. Shows CMV-specific T-cells' cytotoxicity after being expanded in 1G and later exposed to SMG. N=10, MEAN±SEM.

3d. Exposure to 12-hour SMG in a RCCS did not impair in vitro function of γδ-T cells.

Γδ-T cell function was measured against two target cell lines: chronic myeloid leukemia (K562) and multiple myeloma (U266) (Figure 13).

To estimate the effect of exposure to SMG, γδ-T cells that were expanded in 1G were exposed to SMG at the end of the 14-day expansion. SMG exposed γδ-T cells lysed 18.9±4.25% (MEAN±SEM, N=9) of k562 target cells while 1G-control γδ-T cells lysed 19.44±4.5% of them (paired t-test, t(8) =0.5032, p=0.628) (fig. 13f). SMG exposed γδ-T cells lysed 21.41±1.92% (MEAN±SEM, N=9) of U266 target cells while 1G-control γδ-T cells lysed 21.08±2.76% of them (paired t-test, t(8) =0.1479, p=0.886) (fig. 13g).

To estimate the residual effect of exposure to SMG, γδ-T cells that were exposed to SMG and later expanded in 1G for 14-days were used in the killing assays. SMG exposed γδ-T cells lysed 16.44±2.67% (MEAN±SEM, N=9) of k562 target cells while 1G-control γδ-T cells lysed 13.11±2.23% of them (paired t-test, t(8) =1.489, p=0.174) (fig. 13d). SMG exposed γδ-T cells lysed more U266 cells than 1G-control γδ-T cells. Interestingly, SMG-exposed γδ-T cells killed more U266 cells than 1G-exposed γδ-T cells. While SMG-exposed γδ-T cells lysed

23.06±2.86% (MEAN±SEM, N=9) of U266 target cells, 1G-control γδ-T cells lysed only 18.09±2.26% of them (paired t-test, t(8) =2.405, p=0.042) (fig. 13e).

3e. Exposure to 12-hour SMG preserved major subset proportions during a γδ-T cell expansion but induced minor phenotypic changes.

Both conditions showed high purity of γδ-T cells in the expansion product with 96.48±0.67% (MEAN±SEM, N=8) of SMG condition and 95.16±1.5% of the 1G-control condition were identified as γδ-T cells (CD3+ γδ+). Majority of the γδ-T cells were Vγ9Vδ2+ with 87.83±3.16% in the SMG condition and 86.45±3.93% in 1G-control staining +ve for it. NK cell contamination remained low with 2.72±1.49% in the SMG condition and 2.26±0.76% in 1G staining +ve for NK cells (CD3-CD56+). Marginal downregulation of inhibitory receptor CD158b+ (KIR2DL2+) with 27.38±4.36% in 1G-control and 25.81±4.18% in SMG was the only significant phenotypic difference between the conditions (paired t-test, t(7)=2.693, p=0.03). Complete phenotyping shifts are shown in Figure 14.

To estimate the effects of exposure to SMG on γδ-T cells that were expanded in 1G, γδ-T cells that were expanded in 1G were exposed to SMG at the end of the expansion. Further downregulation of inhibitory receptor CD158b+ (KIR2DL2+) was evidenced with only 22.37±3.97% expressing it after exposure to SMG compared to 23.59±4.06% in 1G-control. This downregulation trended towards significance (paired t-test, p=0.05). Upregulation of activating receptor NKG2D was observed with 37.89±7.76% in 1G-control to 48.87±7.15% in SMG condition expressing it (Wilcoxon matched-pairs signed rank test, p=0.0078). Similar

upregulation of Vγ9Vδ1+ γδ-T cells from 1.26±0.59 to 2.51±1.09% was observed (Wilcoxon matched-pairs signed rank test, p=0.0039). Complete phenotypic shifts are shown in Figure 15.

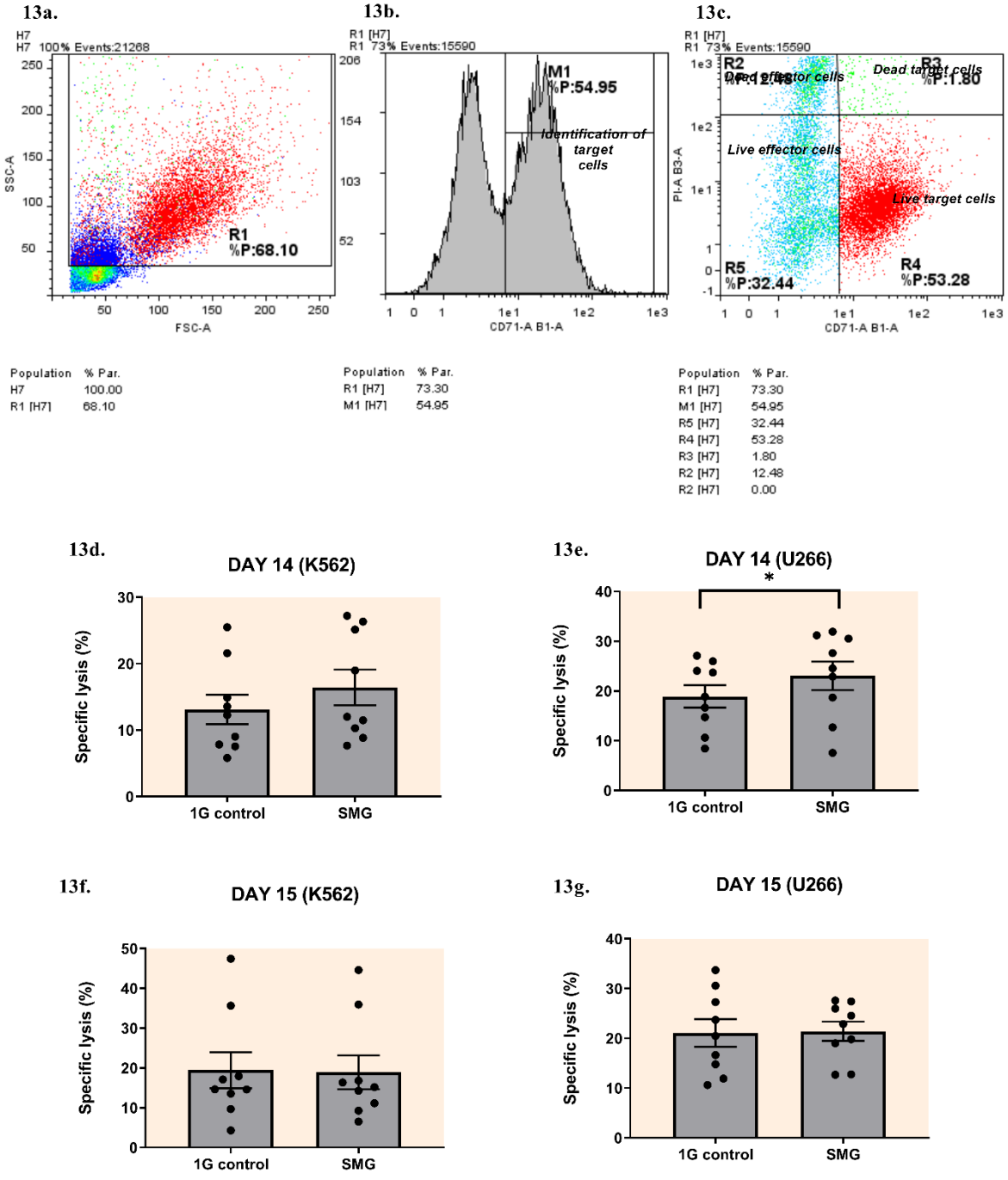
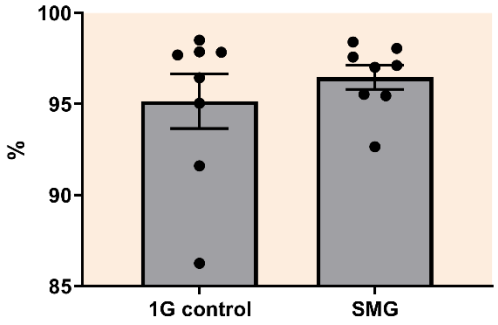


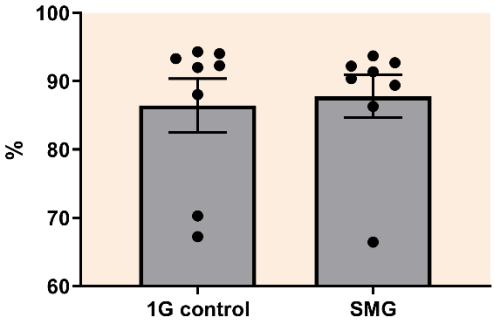
Figure 13. Effect to SMG did not suppress γδ-T cell function. Flow cytometry based killing assay was used to evaluate the function of γδ-T cells (13a-c). Exposure to SMG did not suppress γδ-T cell ability to kill target cells (13f and 13g). N=9, MEAN±SEM, paired t-test, p=0.63 (K562), p=0.88 (U266). Exposure to SMG and later expanding in 1G did not suppress γδ-T cell ability to

kill target cells (13d and 13e). N=9, MEAN±SEM, paired t-test, p=0.17 (K562), p=0.04 (U266). γδ-T cells exposed to SMG and later expanded in 1G killed more multiple myeloma (U266) cells (13e).

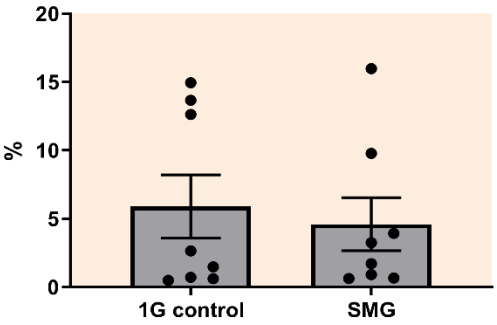
14a.



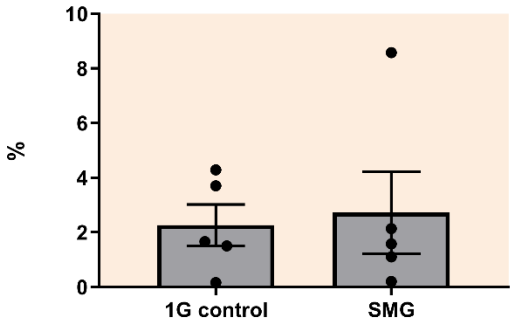
14b.



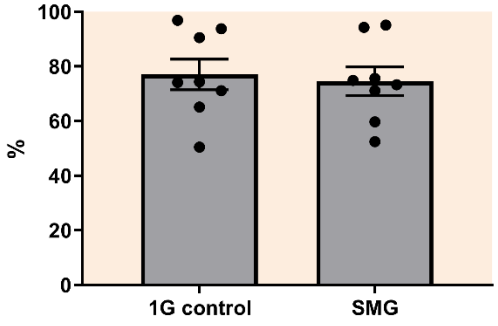
14c.



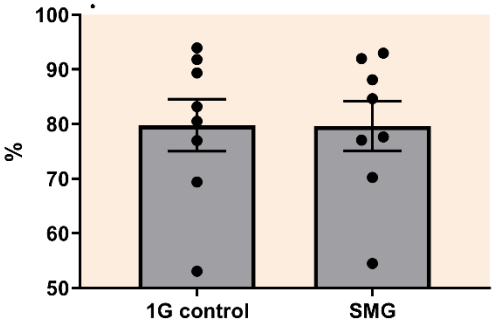
14d.



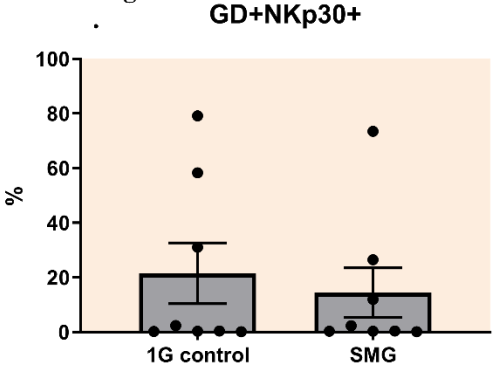
14e.



14f.



14g.



14h.

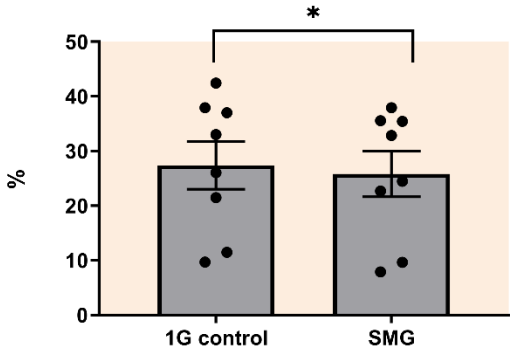
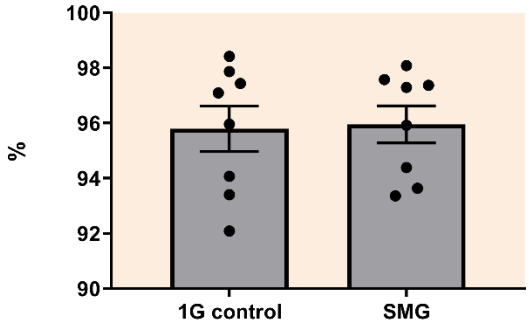


Figure 14. Exposure to SMG did not alter γδ-T cell phenotype in the expanded product at the end of 14-day expansion. γδ-T cell (14a), vγ9vδ2 (14b.), vγ9vδ1 (14c.), NKG2D+ (14d.), NKG2A (14e.), NKp30 (14g.) proportions did not change significantly. Inhibitory receptor CD158b+ (14h.) was significantly downregulated in the expanded product after exposure to SMG (paired t-test, p=0.03). NK cell proportions (14d.) in the expanded product was similar between conditions.

15a.



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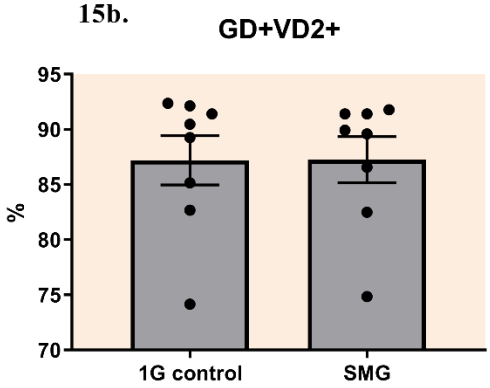
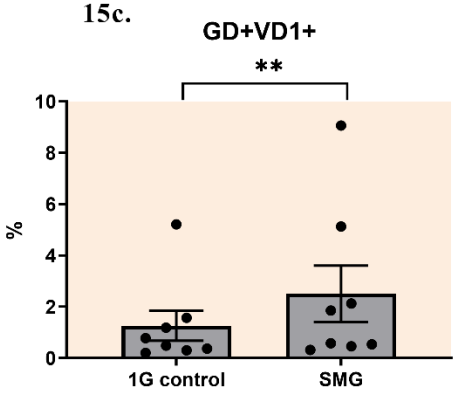
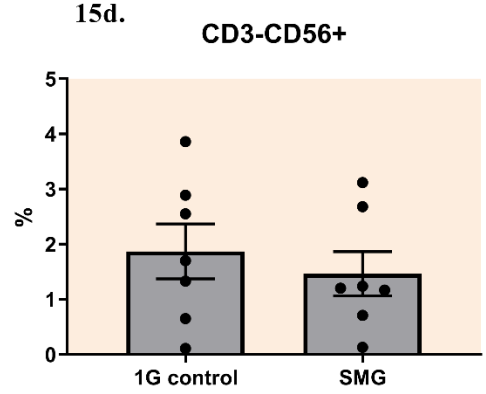


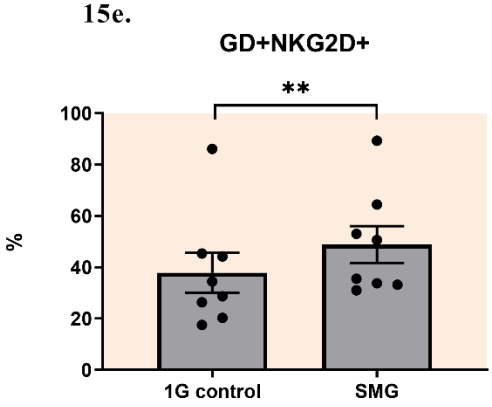
15c.



15d.



15e.



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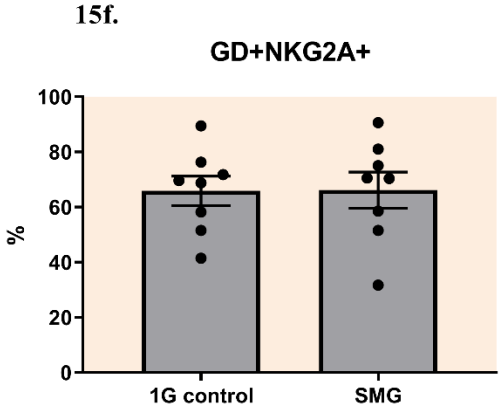
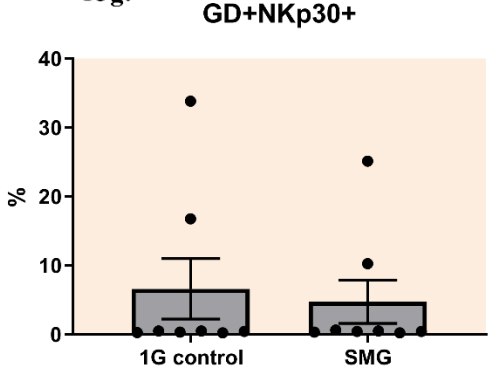


15g.



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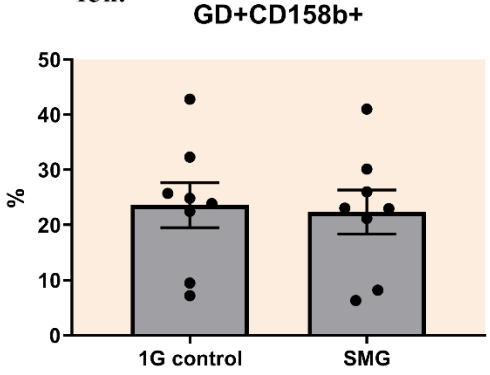

Figure 15. Exposure to SMG induced minor γδ-T cell phenotype changes when 1G-expanded product was exposed to SMG at the end of 14-day expansion. γδ-T cell (15a), vγ9vδ2 (15b.), NKG2A (14e.), NKp30 (14g.) proportions did not change significantly. Activating receptor NKG2D+ (15d.) was upregulated (Wilcoxon matched-pairs signed rank test, p=0.0078). Vγ9vδ1 (15c.) subset showed higher proportions after exposure to SMG (Wilcoxon matched-pairs signed rank test, p=0.0039). NK cell proportions (15d.) did not alter after SMG exposure.

4. Discussion

A significant proportion of effector memory CMV-specific T-cells in CD8+ T-cell compartment has been shown to be beneficial and necessary to control CMV latency. In addition, expanding from smaller proportions to occupy significant portions of the T-cell compartment is vital to control reactivation. This ability becomes important in spaceflight where increased psychological and physiological stress in confluence with various other detrimental factors might threaten a full-blown CMV reactivation in crewmembers on an exploratory mission. Therefore, we examined the effect of exposure to short-term SMG on the expansion potential of CMV-specific T-cells.

The expansion potential of SMG exposed CMV-specific T-cells was suppressed compared to both 1G-controls. At day 14 after exposure to SMG, 10million PBMCs yielded 16 and 10 million fewer CMV-specific T-cells compared to 1G and 1G-rotational controls respectively. This suggests viral specific T-cells are susceptible to the dampening effects of SMG on immune cells.

Hypothesis 1a regarding CMV-specific T cells holds true that exposure to 12-hour SMG in a RCCS impaired in vitro expansion of CMV-specific T cells.

There exists wide variation in subsets and maturation phenotypes specific to CMV in the circulating blood of individuals. This could vary by age, and other characteristics including general immune health and stress levels of the individual during the time of blood draw. This undoubtedly affected the total number of cells in the final expansion product between subjects. Normalization to total CMV-specific viral T-cells was possible due to IFNγ secretion assay

performed at the end of the expansion facilitating enumeration of CMV-specific T-cells in the expansion product. Additionally, using a paired sample experimental design from the same subject controlled for inter-subject variability. Enumeration of CMV-specific T-cells was not performed on PBMCs collected from the subjects at Day 0. The underlying assumption being that there would be similar proportions of CMV-specific T-cells in 1G-control and SMG-exposed PBMCs at the beginning of the expansion. However, this remains a limitation of these experiments. Furthermore, it is unlikely that SMG exposure preferentially depletes viral-specific T-cells from the PBMCs.

CMV-specific T-cells appear to retain their ability to recognize CMV-peptides when presented by antigen presenting cells and then proceed to lyse the cells. Hence, hypothesis 1b. regarding CMV-specific T-cells is disproved.

There was also high variability in both peptide recognition per 10^6 T-cells in ELISPOT data and number of target cells killed per 10^5 CMV-specific CD8 T-cells in the cytotoxicity data. This can also be attributed to variability in various effector and secretory phenotypes of the CMV-specific T-cell repertoire in the peripheral compartment of the individuals.

Short-term immune health also influences the proportion and type of CMV specific T-cells deployed in the peripheral blood compartment. Therefore, further studies on physiological responses of immune system to the stress and stimulus of spaceflight in the CMV T-cell repertoire would benefit the understanding of the magnitude of risk posed by latent viruses during long duration spaceflight.

Past evidence shows spaceflight alters secretory profile of CD4 T-cells towards a TH_2-cytokine shift. There was also a reduction in levels of IL-2 and IFNγ-secreting CD4+ and CD8+ T-cells upon return from both Shuttle and ISS missions (B. E. Crucian, Stowe, Pierson, & Sams, 2008a). Functional assays in this dissertation focused on cytotoxic (CD8+) CMV-specific T-cells. How SMG effects secretory profile of CMV-specific CD4 T-cells remains to be explored in future studies.

In summary, SMG impairs expansion of CMV-specific T-cells while not affecting their ability to recognize and lyse target cells. Future studies should evaluate the effect of SMG on CD4+ T-cell function while also estimating the systemic effect of spaceflight on CMV immune-surveillance and latency control.

Γδ-T cells' ability to expand from a small proportion of T-cells to relatively large numbers when detecting phosphoantigens remains an early design in the adaptive immune system to curtail hematological malignancies at early stages. This function is crucial during spaceflight, since tumor transformation in hematopoietic system is a known risk due to space radiation. Γδ-T cells can work in conjunction with NK cells to curtail any tumor transformation in early stages before adaptive immune system could be geared up. Therefore, we examined the expansion potential of γδ-T cells after being exposed to SMG.

The expansion potential of SMG exposed γδ-T cells appears to be lagging behind 1G-control. At day 14 after exposure to SMG, there were 11million more γδ-T cells in 1G-control condition. This accounted for 10% fewer γδ-T cells in SMG condition. Hypothesis 1a

regarding γδ-T cells holds true that exposure to 12-hour SMG in a RCCS impaired in vitro expansion of γδ-T cells.

Their ability to expand could be recovering over a 14-day expansion in our experimental design. This might be due to the exposure to SMG being limited to 12-hours while the rest of the 14-day expansion occurs in 1G. It would be worthwhile to investigate whether they expand as well when the expansion occurs in microgravity over extended periods. While the expansion potential of SMG exposed cells appears to be lagging behind 1G-control; their ability to expand seems to recover over a 14-day expansion. An interesting question would be to investigate whether they expand as well when the expansion occurs in microgravity.

Another major risk with space radiation are epithelial tumors due to high turnover rates in epithelial cells. Vγ9Vδ1 subset of γδ-T cells reside in the intraepithelial chambers of gut mucosa, and skin and detect stress ligands (PAMPs). This expansion protocol was primed to expand Vγ9Vδ2 subset of γδ-T cells, which primarily exist in the vascular compartment. Future studies could look at the specific effect of SMG exposure on tissue vigilance of Vγ9Vδ1 cells.

Γδ-T cell function appears to be sustained after exposure to SMG. Their alternative activation mechanisms might be the reason behind their resistance to SMG-induced suppression in functionality. While immediate exposure to SMG did not appear to affect γδ-T cell function (figures 13f. and 13g.), pre-expansion exposure to SMG appears to have increased γδ-T cell

function. While there was non-significant increase in specific lysis against chronic myeloid leukemia (K562) cells (fig.13d), there was a significant increase in killing against multiple myeloma (U266) cells (fig. 13e). This could be explained by minor downregulation of inhibitory receptor CD158b+ expressing γδ-T cells (fig.14h).

Immediate changes in γδ-T cell receptor phenotype included upregulation of activating receptor NKG2D (fig.15e) and a trend towards further downregulation of inhibitory receptor CD158b+ (fig.15h). However, these changes did not translate to any functional differences in killing assays possibly due to the minute effect sizes of these changes and their resultant effect on γδ-T cell function. It is also possible that there was a saturation of NK receptor signaling that determines γδ-T cell response. Further increase in function might need alternative activating and killing mechanisms. In summary, single exposure to 12-hour SMG in a RCCS does not impair in vitro function of γδ-T cells disproving hypothesis 1b regarding γδ-T cells.

NK cells appear to be more susceptible to SMG-induced suppression in function as previously reported (Mylabathula et al., 2020). NK cell killing is restricted by the balance between activating/inhibitory signals from the NK-cell receptors. This determines the outcome of an interaction with a suspicious cell. Fortunately, γδ-T cells have alternative γδ-TCR-peptide-MHC interaction in addition to NK-cell receptor signaling, which supplements the decision-making.

In summary, SMG impairs γδ-T cell expansion while not hindering γδ-T cell function. Future studies should evaluate the effect of Vγ9Vδ1 subset of γδ-T cell function along with their interaction with other immune subsets.

Chapter 5

Effect of simulated microgravity on *in vivo* anti-leukemia activity

1. Introduction

NK cells are crucial subset of innate immune system that specialize in identifying tumor transformed cells and virus-infected cells. They rely on their activating and inhibitory signals and their interaction with MHC class I molecules to identify erratic cells in our body (Orr & Lanier, 2010; Topham & Hewitt, 2009). MHC receptor signal perturbations are one of the early signs of tumor transformation or viral infection of a cell (Garcia-Lora, Algarra, & Garrido, 2003; Hewitt, 2003). Therefore, NK cells have been ascribed the role of preventing tumor transformation and controlling tumor growth in early stages. We have previously shown that NK cells kill 50% fewer target cells *in vitro* after exposure to SMG for 12-hours (Mylabathula et al., 2020). Fewer NK cells expressed perforin and granzyme B. They also showed lower levels of degranulation and effector cytokines (TNFα and IFNγ) production when encountering target cells. Furthermore, cancer cells (K562) did not show any changes in susceptibility to NK cell killing. This suggests that while innate immune cells might be disarmed by microgravity, tumor cells might exhibit a certain level of resistance to the detrimental effects of microgravity. This might tip the scale in favor of tumorigenesis in spaceflight, especially when increased exposure to stimuli like space radiation is taken into account.

While *in vitro* experiments help us to evaluate immune cell function in a strictly controlled environment, translating this to an *in vivo* setting will help us to estimate the effect of any stimulus in a physiologically more relevant environment. Additionally, the true magnitude of impairment of immune cell's efficacy after exposure to microgravity will be better estimated in an *in vivo* model. In addition to function, these models could test an immune cell's

surveillance, homing, extravasation, tumor-infiltration properties which are all crucial to prevent disease and prolong health.

Therefore, this dissertation examined if the impairment of *in vitro* NK cell function after exposure to SMG will also extend to an *in vivo* model. We used immunocompromised NSG-tg(hu-IL15) mice engrafted with chronic myeloid leukemia (K562) and evaluated the differences in tumor growth control between human immune cells that have been exposed to either 1G or simulated microgravity.

2. Experimental Design

To evaluate tumor growth control of primary human NK cells, we optimized the model to prevent the effect of other immune cells from modulating the tumor growth. Most of the anti-tumor activity in naïve PBMCs can be approximated to NK cells (Kandarian, Sunga, Arango-Saenz, & Rossetti, 2017). Moreover, using PBMCs for *in vitro* cytotoxicity assays provides better estimation of *in vivo* activity since NK cells do not act as an isolated group of cells in the body (Park et al., 2013; Sierich & Eiermann, 2013).

Initial experiments showed that around day 28 (4weeks) after injection with 10 million PBMCs, the mice showed signs of moderate GVHD. The signs commonly included hunched posture indicating intestinal distress, ruffled fur and naked/scaly skin indicating distress of skin, reduced activity indicating general malaise, and occasional diarrhea. Another sign included weight loss. Weight loss of more than 10% initial weight was evaluated as a GVHD/morbidity score of 1. Weight loss greater than 20% indicated that the mouse has reached sacrificial criteria in accordance with our IACUC guidelines. Since GVHD has known immune-modulatory effects, we stopped estimating anti-leukemia activity after moderate GVHD set in. This was a GVHD score of 4 or 5 within a total possible score of 10.

'TUMOR control' mice, which did not receive human immune cells, were piloted to study unrestrained tumor growth dynamics. Using 1 million K562 cells tagged with luciferase initially showed luminescence levels at 1-8e^6 photons/sec during the first week. After some minor fluctuations, there was a steady exponential growth in tumor burden represented by in

vivo bioluminescence intensity (BLI) (measured in photons/second). Tumor bioluminescence reached saturation levels of $1e^{10}$ to $1e^{11}$ around 6 weeks after tumor cells injection.

Therefore, the experiment was designed for a duration of 6 weeks, with human PBMCs injected one week after tumor cells injection. This allowed time for the tumor cells to be established in the animal's body. Their growth, in the presence of human immune cells, was monitored via bioluminescent imaging. Any reduction in bioluminescence compared to 'TUMOR control' can be attributed to anti-leukemia activity of effector cells in the PBMCs.

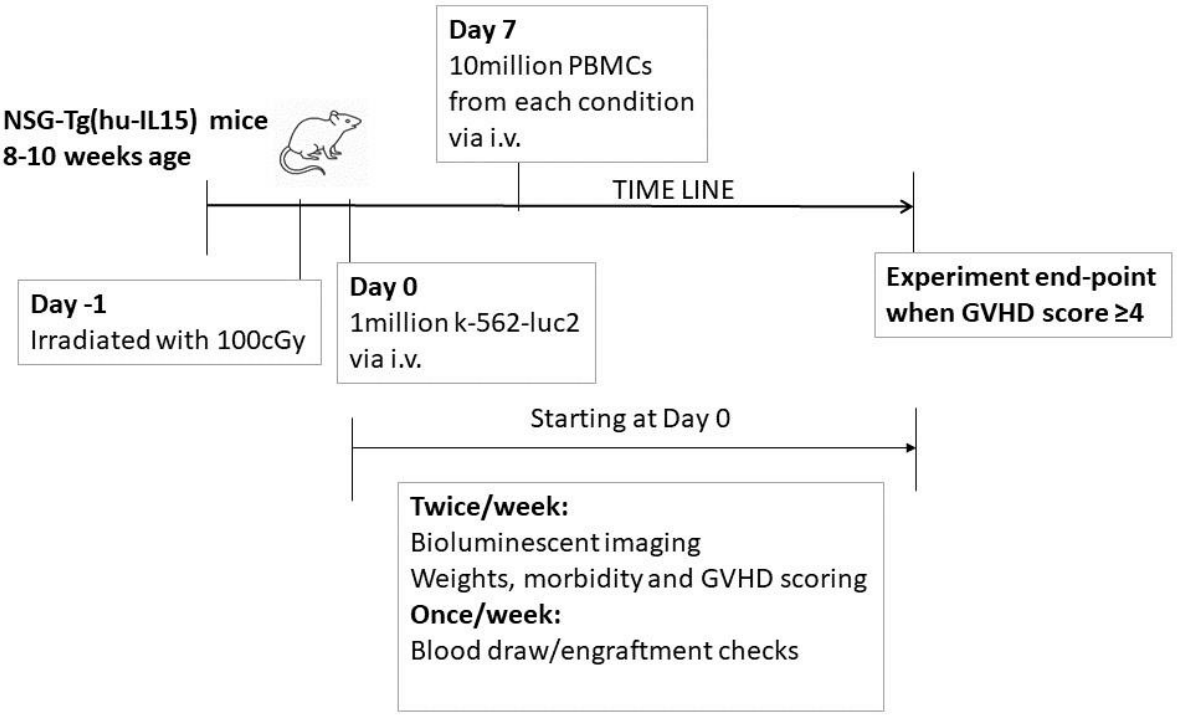

Figure 16. Experimental design for AIM 2.

Hypothesis 2[4]: NSG-tg(hu-IL15) mice were injected with 1 million luciferase-labelled human leukemia cells (K562) and one week later 1 million human PBMCs exposed to SMG, rotational control and STATIC conditions were engrafted into the mice. Anti-leukemia activity was measured as reduction in bioluminescent intensity (BLI) score in comparison to 'TUMOR control' mice. 1G-control and SMG conditions were compared within each experiment that had PBMCs from the same human participant controlling for differences in immune cell subsets. They also received same batch of K562 controlling for differences in tumor growth

[4] Exposure to 12-hour SMG in a RCCS adversely affects anti-leukemia activity of human immune cells in vivo by impairing their ability to control tumor growth in a NSG-tg(hu-IL15) mice model.

due to viability and number of passages in cell culture. Experiment was ended when severe GVHD starts setting in (composite GVHD score≥4). This was done to prevent immunomodulatory effects of GVHD from making it complicated to evaluate the *in vivo* anti-leukemia activity of effector lymphocytes. Separate mice with PBMCs only and without k-562-luc2 tumor were used to check for engraftment levels amongst the conditions to prevent different levels of lymphocyte engraftment being a confounding factor. This enabled us to determine if SMG exposed lymphocytes have diminished survival *in vivo*. Timeline of tumor and PBMC injections (figure 16): One million K-562-luc2 cell were administered intravenously via a tail vein injection after irradiating the mice with 100cGY using a cesium-32 irradiator on the previous day. One week later, normal saline (TUMOR control) or 10million PBMCs from respective conditions (1G-CONTROL or SMG) were injected into the mice. Progression of tumor was monitored after injecting 200µl of luciferin (15mg/ml) intra-peritoneally. Mice were anesthetized with 2% isoflurane and BLI scores (photons/second) were obtained using LagoX spectral imager.

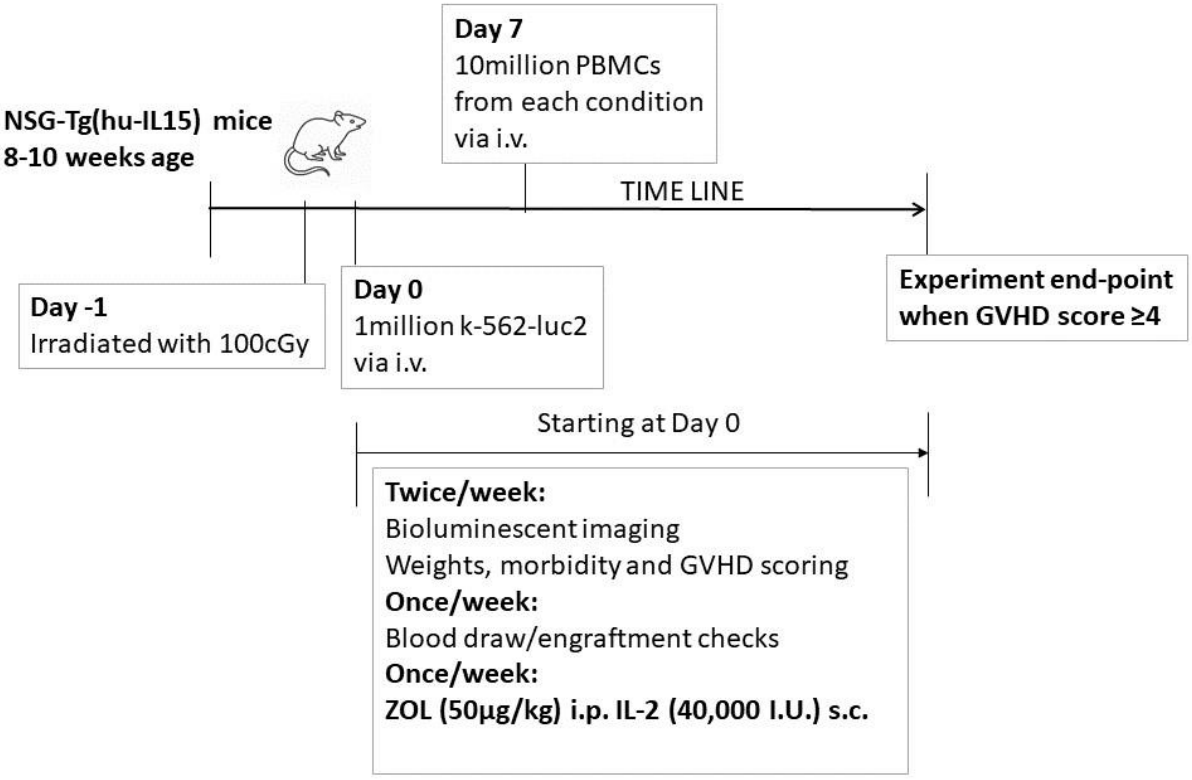

Figure 17 Experimental design for AIM 3.

Hypothesis 3[5]**:** In an attempt to improve immune-function in SMG-exposed PBMC group, ZOL (50µg/kg) intraperitoneally (Ottewell et al., 2008) and IL-2 (40,000 I.U.) subcutaneous (Himizu, Ields, & Iedlin, 1999) were administered once/week (figure 17).

[5] Systemic administration of ZOL+IL-2 will boost the in vivo anti-leukemia capacity of human immune cells in an NSG-tg(hu-IL15) mice model and/or in an in vitro model.

For hypotheses 2 and 3, Bioluminescent imaging (BLI) score (**photons/second**) which is a measure of **tumor burden** was used as dependent variable with onset of severe GVHD and tumor saturation as termination points for the experiment.

3. Results

Hypothesis 2:

3a. Exposure to SMG impaired anti-leukemia activity of human immune cells *in vivo*.

Tumor growth control was compared between mice that were injected with PBMCs exposed to SMG (TUMOR+SMG PBMCs) or 1G-control (TUMOR+1G PBMCs) to evaluate the effect of SMG on anti-leukemia activity of human immune cells *in vivo*. TUMOR control was used as a reference for unrestrained tumor growth. Representative BLI images showing tumor dynamics *in vivo* are shown in Figure 18. A mixed effects model was used to analyze BLI scores with 'condition' (TUMOR control, TUMOR+SMG PBMCs, TUMOR+1G PBMCs) and 'time' as main effects and an interaction term 'condition*time' in the model.

Time, condition and time*condition effects were all statistically significant ($p<0.0001$) (fig.19a). Significant interaction effect suggested that 'tumor burden' grew differentially over time in different conditions. A pairwise comparison indicated that there was significant interaction ($p<0.0001$) between TUMOR+1G PBMCs and TUMOR+SMG PBMCs revealing that 1G-exposed PBMCs controlled tumor growth better than SMG-exposed PBMCs. There was also significant interaction ($p<0.0001$) between TUMOR+1G PBMCs and TUMOR control conditions as expected showing 1G-exposed PBMCs ability to control tumor growth. Interestingly, there was no interaction ($p=0.082$) between TUMOR+SMG PBMCs and TUMOR control, showing that while SMG-exposed PBMCs might have shown some anti-

leukemia activity, the tumor burden in mice with SMG-exposed PBMCs was not curtailed significantly compared to mice that only received tumor.

Peak BLI reached during the experiment was also compared between the conditions using matched pairs non-parametric Friedman test (fig.19b). After a significant effect in the model ($p=0.0018$), a post-hoc comparison was performed. Dunn's multiple comparison test revealed that TUMOR+1G PBMCs reached a significantly lower BLI score during the experiment compared to both TUMOR control ($p=0.003$) and TUMOR+SMG PBMCs ($p=0.013$). TUMOR+SMG PBMCs did not show a lower peak BLI during the experiment compared to TUMOR control ($p>0.99$) further emphasizing SMG-exposed PBMCs inability to control tumor growth.

3b. Exposure to SMG did not alter survival in mice injected with SMG PBMCs.

To ensure differential survival was not a confounding factor between the conditions over time, a survival analysis was done. . A log-rank (Mantel-Cox) test was used for survival analysis. As expected, survival did not significantly differ between conditions ($\chi2=2.934$, $p=0.231$) (fig. 19c).

During the 6 weeks duration of the experiment, there were more deaths in TUMOR control (deaths=4) compared to both TUMOR+SMG PBMCs (deaths=3) and TUMOR+1G PBMCs

(deaths=1) among 12 mice in each group. Most of the deaths occurred in the later part of the experiment in correspondence with tumor burden.

3c. Exposure to SMG did not alter GVHD/morbidity severity.

The duration of the experiment was limited to 6 weeks to prevent differential survival and GVHD from confounding the analysis. GVHD/morbidity onset, progression, and severity were monitored between both conditions that received PBMCs. A linear mixed model was used to analyze GVHD scores overtime with 'condition' (TUMOR control, TUMOR+SMG PBMCs, TUMOR+1G PBMCs) and 'time' as main effects. An interaction term 'condition*time' was also introduced into the model.

Significant time effect was observed as GVHD/morbidity scores grew overtime ($p<0.0001$). Neither 'condition' ($p=0.764$) nor 'condition*time' ($p=0.981$) were significant showing that onset, progression and severity between the groups did not differ over time (fig. 19d).

3d. Exposure to SMG did not impair human immune cell engraftment *in vivo*.

To verify that exposure to SMG did not impair PBMCs' ability to thrive later in a xenograft; mice were injected only with PBMCs without tumor. Engraftment was measured as a proportion of CD45human+ (CD45h+) cells in total immune cells in mice blood. SMG did not alter engraftment dynamics of PBMCs (figure 21).

A linear mixed model was used to analyze engraftment overtime with ‘condition’ (TUMOR control, TUMOR+SMG PBMCs, TUMOR+1G PBMCs) and ‘time’ as main effects. An interaction term ‘condition*time’ was also introduced into the model. No significant differences were observed between groups in engraftment of human immune cells (CD45h+%, p=0.175) (CD45h+ cells/uL, p=0.628) (fig. 21a, 21c.) and NK cells (CD45h+ CD3- CD56+%, p=0.219) (CD45h+ CD3- CD56+ cells/µl, p=0.478) (fig. 21b, 21d.). Initial weeks showed high NK cell proportions in blood, with 25.85% in SMG-exposed PBMCs and 23.86% in 1G-exposed PBMCs of the CD45h+ cells being NK cells. Engraftment rose steadily as the experiment progressed with NK cell proportions decreasing as a total proportion of CD45h+ cells. NK cell proportions reached 3.94% in SMG-exposed PBMCs and 1.49% in 1G-exposed PBMCs at week 5. Similar human immune cell engraftment dynamics were observed in mice that had tumor (figure 20).

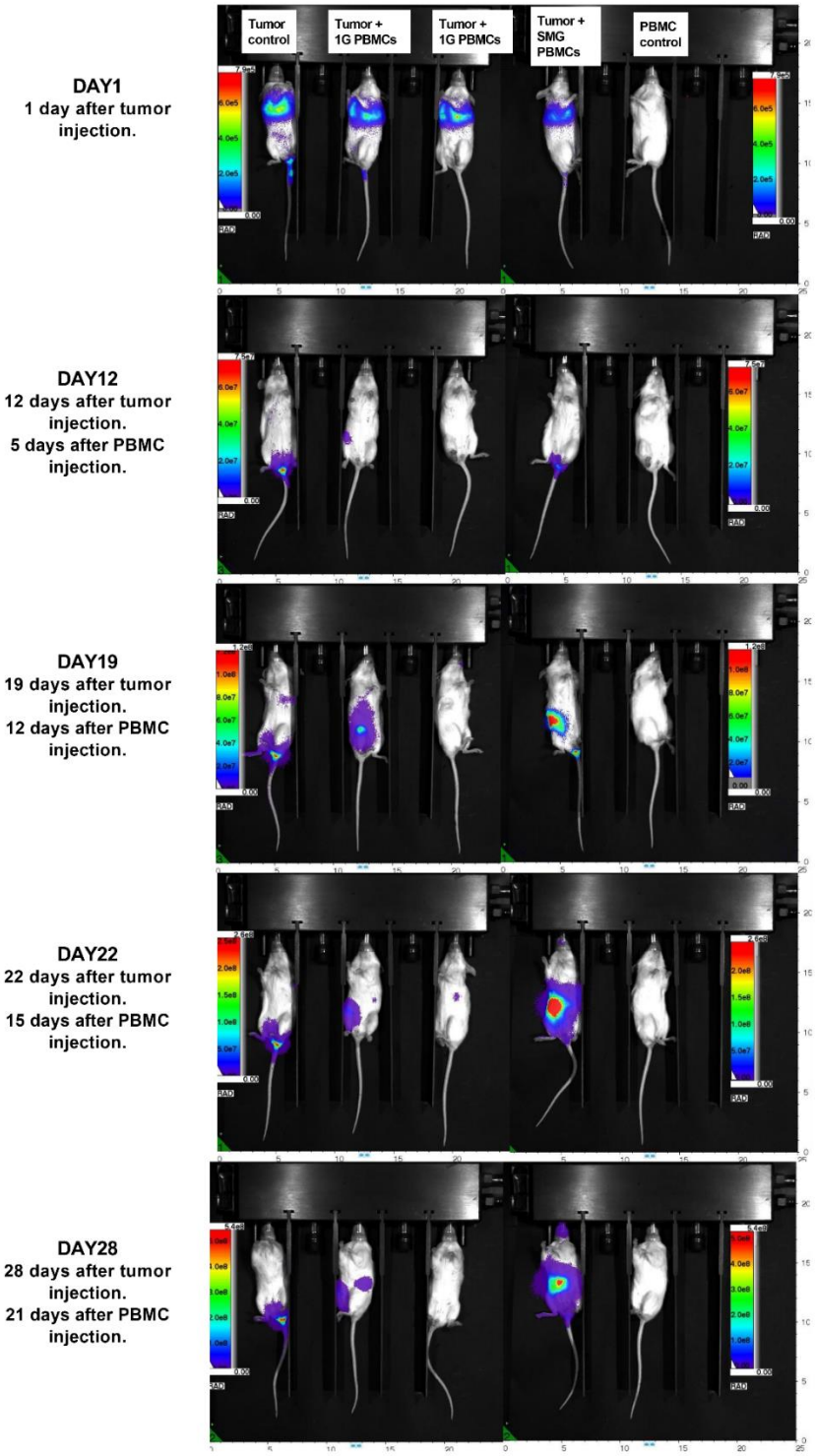

Figure 18. Bioluminescent intensity (BLI) images. Days 1, 12, 19, 22 and 28 of the experiment. Lane 1: tumor control, lanes 3, 5: tumor+ 1G PBMCs, lane 6: tumor+ SMG PBMCs, lane 8: PBMC control.

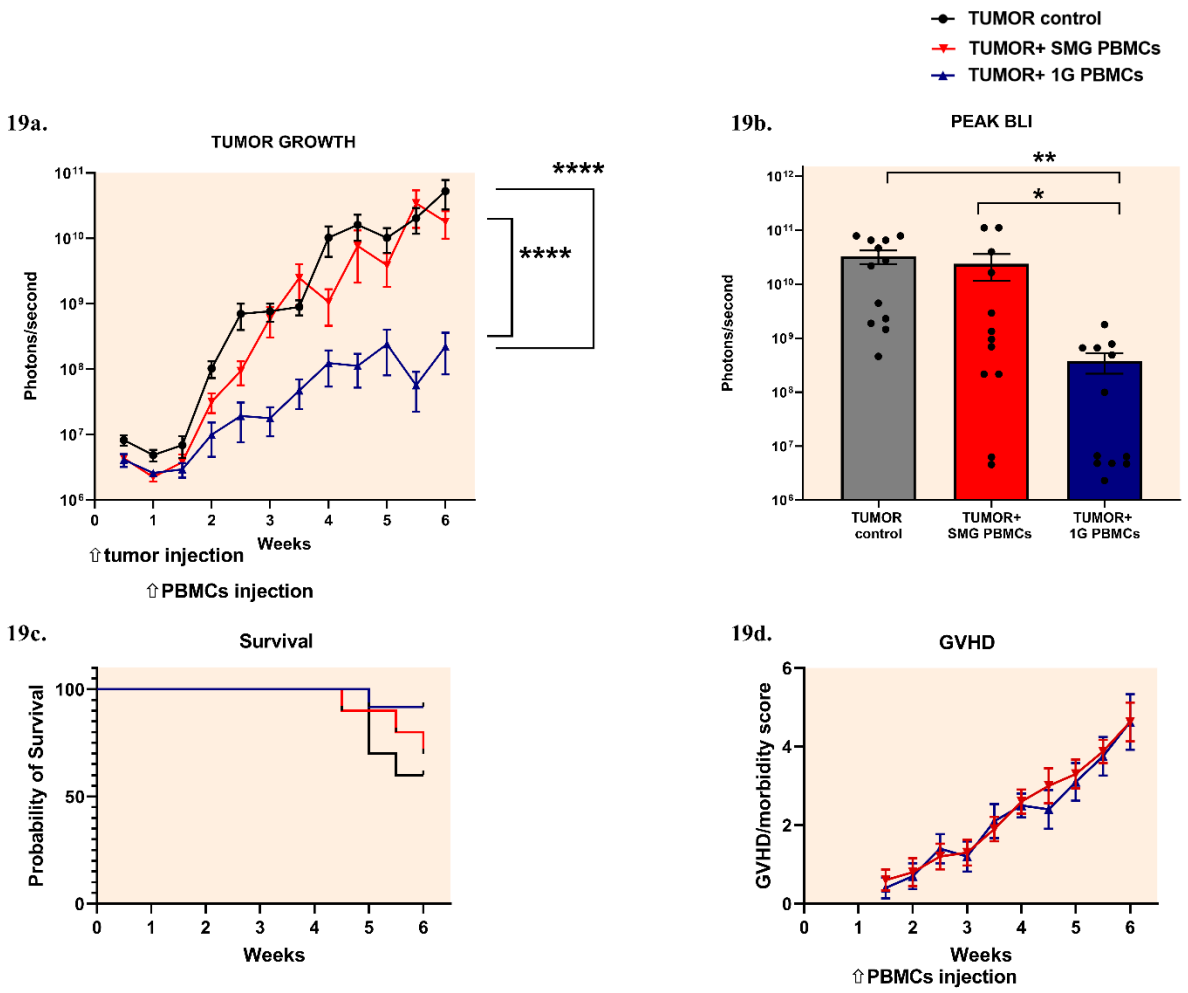

Figure 19. Effect of SMG on effector immune cell function *in vivo*. Exposure to SMG impaired effector immune cell ability to control tumor growth in an *in vivo* model (19a.) (**=p<0.0001). Exposure to SMG also significantly increased peak BLI score during the experiment (19b.) (* denotes p<0.05, ** denotes p<0.01). Exposure to SMG did not affect survival (19c.) and GVHD incidence (19d.). N=12, MEAN±SEM.**

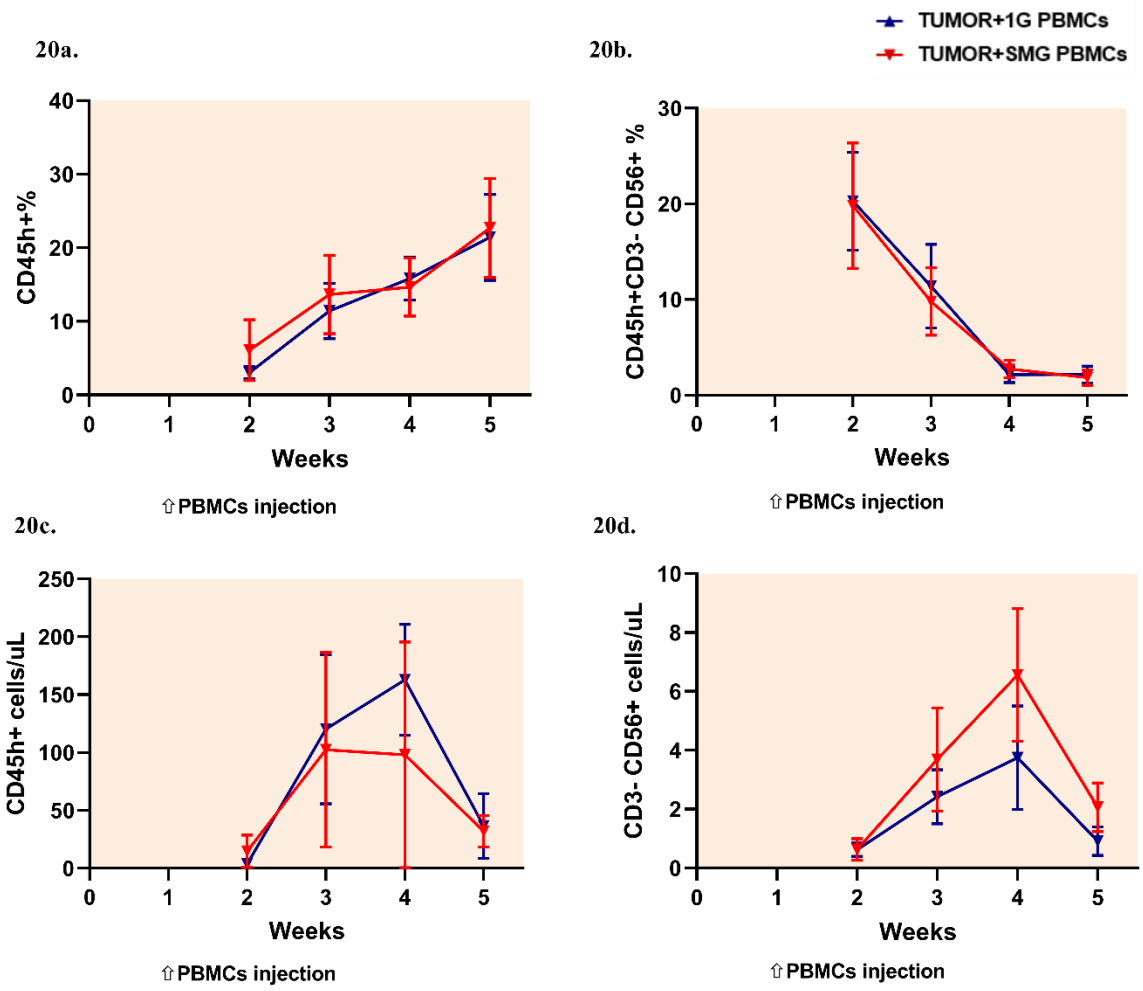

Figure 20. Effect of exposure to SMG on human cell engraftment dynamics *in vivo* in the presence of tumor. Exposure to SMG did not impair human PBMCs' (20a., 20c.) and NK cells' (20b., 20d.) ability to engraft in the presence of tumor. N=10, MEAN±SEM.

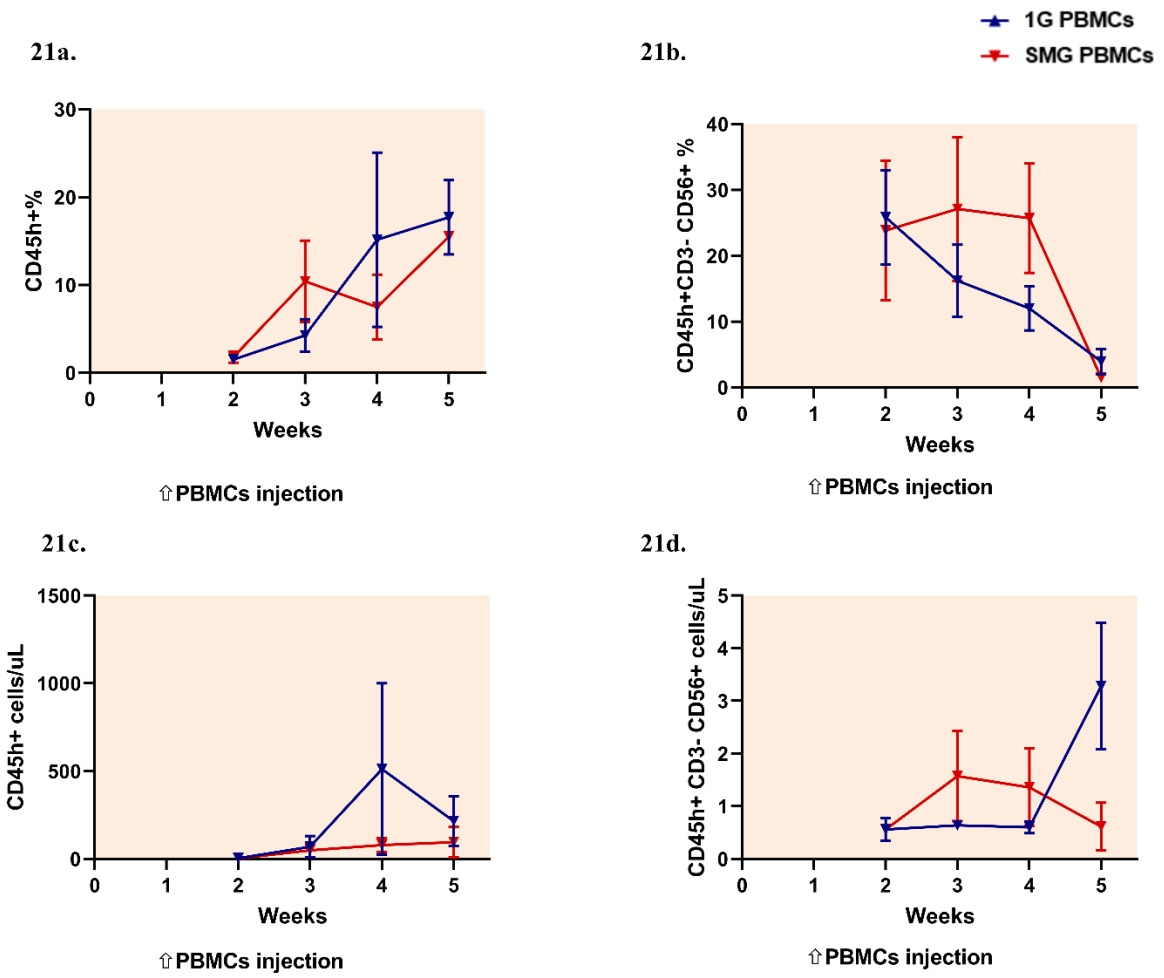

Figure 21. Effect of SMG on human cells engraftment *in vivo* in the absence of tumor. Exposure to SMG did not impair human PBMCs' (21a., 21c.) and NK cells' (21b., 21d.) ability to thrive *in vivo*. N=5, MEAN±SEM.

Hypothesis 3:

3e. Zoledronic acid and IL-2 therapy improved anti-leukemia activity of human immune cells *in vivo*.

In an attempt to boost anti-leukemia activity, ZOL+IL2 therapy was administered to a group of mice that were injected with TUMOR+SMG PBMCs. A mixed effects model was used to analyze BLI scores with 'condition' (TUMOR control, TUMOR+ZOL+IL2 control, TUMOR+1G PBMCs, TUMOR+SMG PBMCs, TUMOR+SMG PBMCs+ZOL+IL2) and 'time' as main effects and an interaction term 'condition*time' in the model.

Time ($p<0.0001$), condition ($p=0.0006$) and time*condition ($p<0.0001$) effects were all significant (fig. 22a) in the complete model. A pairwise comparison indicated that there was significant interaction ($p=0.0004$) between TUMOR+SMG PBMCs and TUMOR+SMG PBMCs+ZOL+IL2 revealing that administration of ZOL+IL2 improved control of tumor growth rate in PBMCs exposed to SMG. TUMOR+SMG PBMCs+ZOL+IL2 did not show an interaction effect with TUMOR+1G PBMCs ($p=0.567$) revealing that ZOL+IL2 administration enabled SMG-exposed PBMCs to control tumor growth rate as efficiently as 1G-exposed PBMCs. There were no significant differences in condition ($p=0.947$) or time ($p=0.221$) between these groups further showing that tumor control patterns were similar between the groups.

TUMOR+1G PBMCs, TUMOR+SMG PBMCs+ZOL+IL2 showed significant interaction with TUMOR control (both $p<0.0001$) showing they effectively controlled tumor growth, while

TUMOR+SMG PBMCs did not show significant interaction with TUMOR control (p=0.381) following the pattern seen in previous experiment, showing SMG-exposure impaired anti-leukemia activity of human immune cells. TUMOR+SMG PBMCs also showed significant interaction (p<0.0001) with TUMOR+1G PBMCs, also following the pattern seen in previous experiment. TUMOR control and TUMOR+ZOL+IL2 showed no differences between groups. Neither condition (p=0.635) nor interaction (p=0.924) were significant between the groups, showing that administration of ZOL+IL2 did not alter tumor growth pattern in the absence of human PBMCs.

Peak BLI reached during the experiment was also compared between the conditions using matched pairs non-parametric Friedman test (fig.22b). After a significant effect in the model (p=0.0076), a post-hoc comparison was performed. Dunn's multiple comparison test revealed that TUMOR+1G PBMCs reached a significantly lower BLI score during the experiment compared to TUMOR+ZOL+IL2 (p=0.027). No other post-hoc comparisons were significantly different from each other.

3f. Zoledronic acid and IL-2 therapy did not alter survival.

ZOL+IL2 therapy did not alter survival ($\chi 2$=2.429, p=0.6575) (fig. 22b). TUMOR control had one death (1/4) during the experiment. 'TUMOR+SMG PBMCs+ZOL+IL2' group had one death (1/5) during the experiment compared to TUMOR+1G PBMCs and TUMOR+SMG PBMCs groups, which had no deaths.

3g. Zoledronic acid and IL-2 therapy did not alter GVHD/morbidity severity.

Administration of ZOL+IL2 therapy did not alter the onset, severity, and progression of GVHD/morbidity (p>0.999) (fig. 22c).

3h. Zoledronic acid and IL-2 therapy did not alter levels of NK and γδ-T cells in blood.

To examine if ZOL+IL2 therapy increased blood levels of NK cells and γδ-T cells, engraftment checks were done weekly similar to the previous experiment. ZOL+IL2 therapy did not alter engraftment dynamics of PBMCs (figure 23). A mixed effects model was used to analyze engraftment overtime with 'condition' (TUMOR control, TUMOR+SMG PBMCs, TUMOR+1G PBMCs) and 'time' as main effects. An interaction term 'condition*time' was also introduced into the model. ZOL+IL2 therapy did not alter engraftment dynamics of total human immune cells (CD45h+%, p=0.713) (CD45h+ cells/uL, p=0.419) (fig. 23a, 23b), NK cells (CD45h+ CD3- CD56+%, p=0.99) (CD45h+ CD3- CD56+ cells/uL, p=0.941) (fig. 23c, 23d), or γδ-T cells (CD45h+ CD3+ γδ-TCR+%, p=0.915) (CD45h+ CD3+ γδ-TCR+ cells/uL, p=0.40.937) (fig. 23e, 23f). ZOL+IL2 therapy showed non-significant increase in NK cells and γδ-T cell proportions and absolute counts throughout the experiment hinting at improved *in vivo* expansion in response to the treatment.

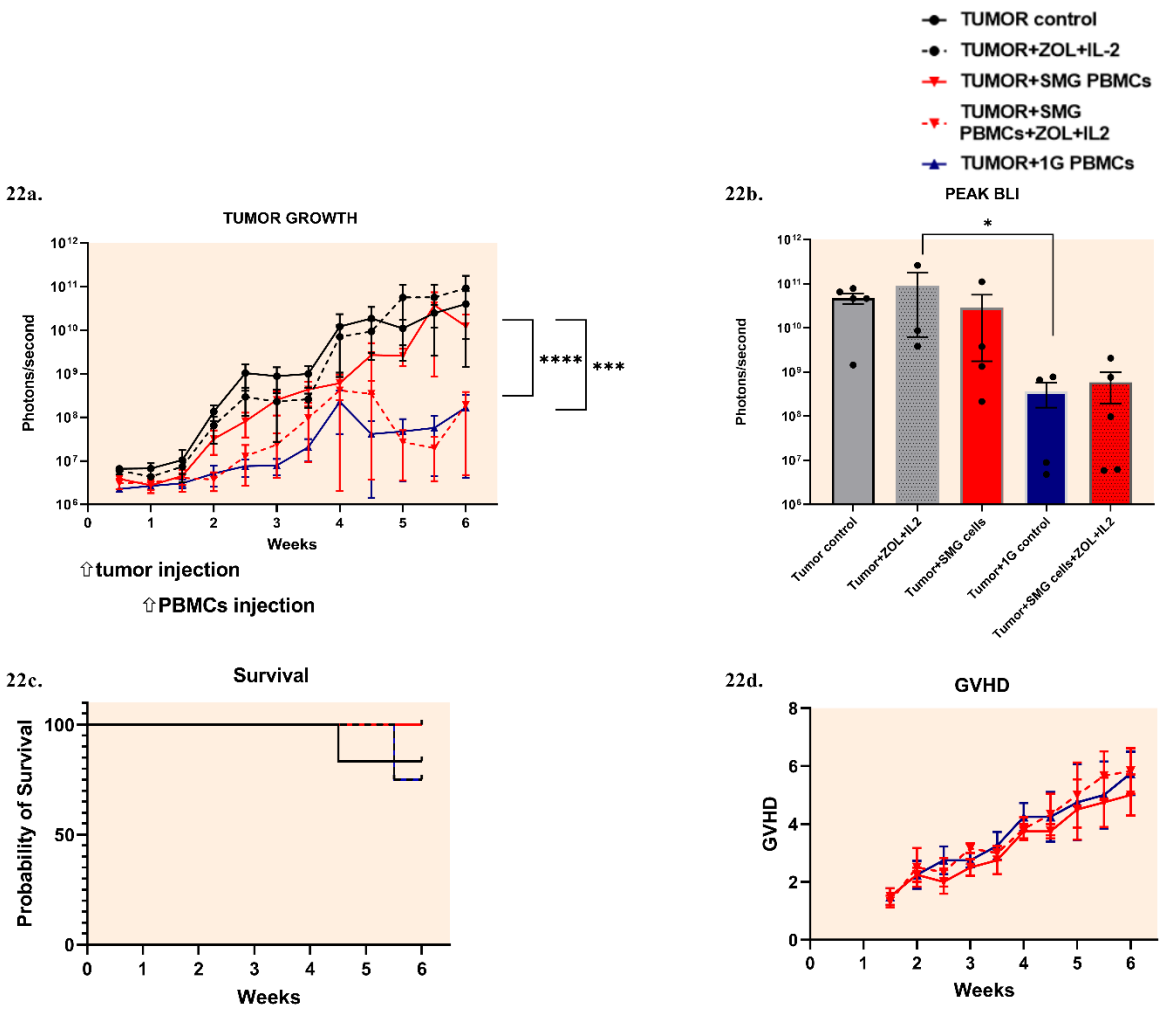

Figure 22. Effect of zoledronic acid+IL2 (ZOL+IL2) therapy on SMG induced suppression of *in vivo* anti-leukemia activity. ZOL+IL2 therapy abrogated SMG induced loss of anti-leukemia activity *in vivo* (22a.), did not affect peak BLI (22b.), survival (22c.), GVHD incidence (22d.). N=5, MEAN±SEM.

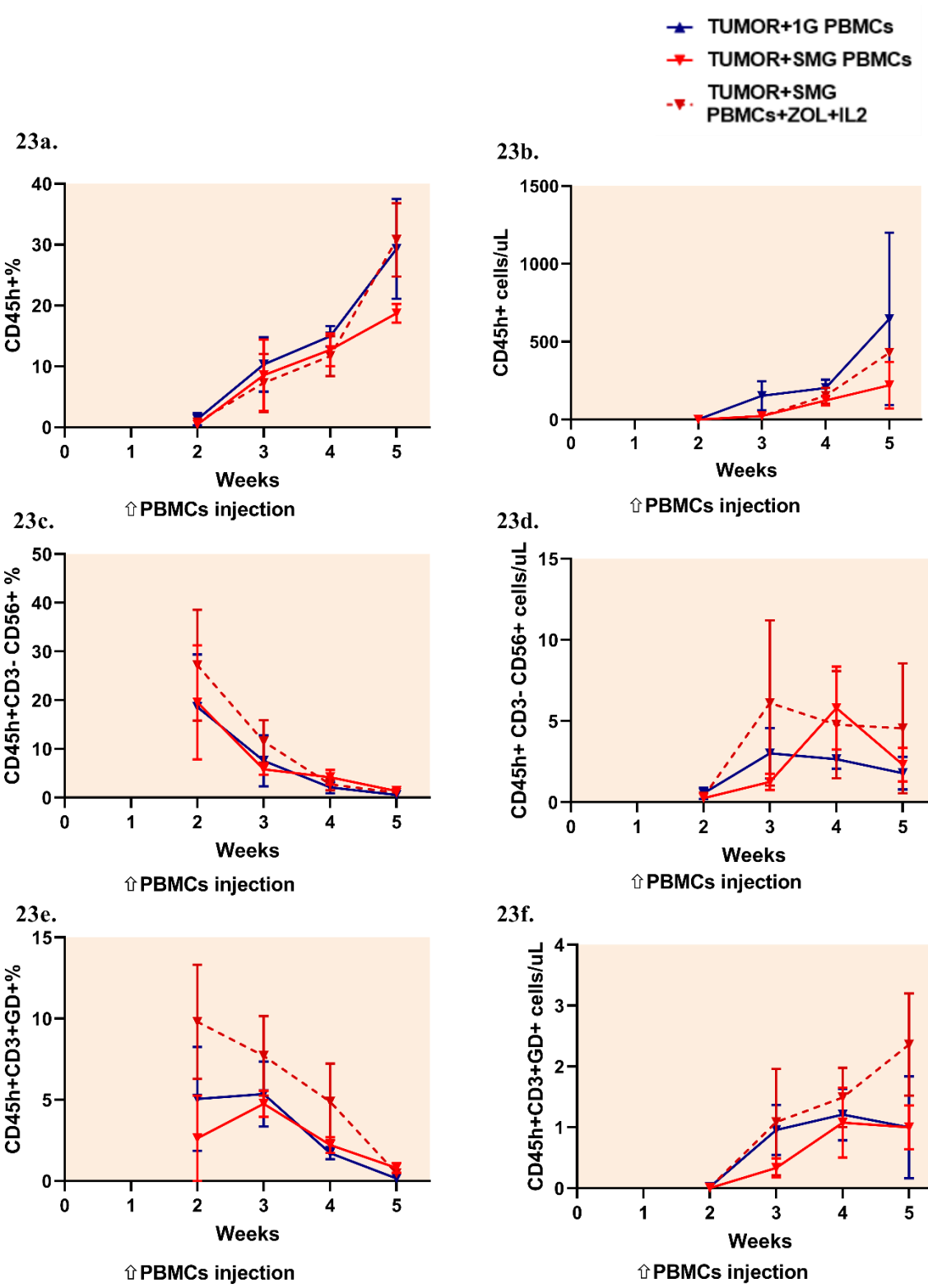
TUMOR+1G PBMCs
TUMOR+SMG PBMCs
TUMOR+SMG PBMCs+ZOL+IL2
23a.
CD45h+%
Weeks
⇧PBMCs injection
23b.
CD45h+ cells/uL
Weeks
⇧PBMCs injection
23c.
CD45h+CD3- CD56+ %
Weeks
⇧PBMCs injection
23d.
CD45h+ CD3- CD56+ cells/uL
Weeks
⇧PBMCs injection
23e.
CD45h+CD3+GD+%
Weeks
⇧PBMCs injection
23f.
CD45h+CD3+GD+ cells/uL
Weeks
⇧PBMCs injection

Figure 23. Effect of SMG and ZOL+IL2 therapy on human cells engraftment in vivo in the presence of tumor. ZOL+IL2 therapy did not alter human PBMCs' (23a., 23b.) and NK cells' (23c., 23d.) and γδ-T cell (23e., 23f.) engraftment dynamics. N=5 MEAN±SEM.

4. Discussion

The main aim of this dissertation was to examine if suppression in NK cell function due to SMG exposure (previously observed *in vitro*) would translate into an *in vivo* setting. In a 6-week experimental model to estimate *in vivo* function, anti-leukemia activity was evaluated as reduction in bioluminescence intensity compared to mice that have been injected with only tumor cells. The main result reported here is that impairments in effector lymphocyte anti-leukemia activity extend to *in vivo* setting.

SMG-exposed PBMCs showed impaired anti-leukemia activity *in* vivo, as evidenced by poor control of tumor growth rate compared to SMG-exposed PBMCs. TUMOR+SMG PBMCs mice showed tumor burden increase at the same rate as TUMOR control resulting in same aggregate tumor levels ($1e^{10}$-$1e^{11}$) at the end of the experiment. TUMOR+1G PBMCs mice had much better control over tumor growth. Their BLI scores were consistently lower; with their tumor burden at 6 weeks not rising above $1e^{8}$-$1e^{9}$. Peak BLI scores were also significantly lower in TUMOR+1G PBMCs group compared to TUMOR+SMG PBMCs group. These experiments show that exposure to SMG impaired immune cell ability to control tumor growth rate *in vivo*. While tumor lysis in a 4-hour *in vitro* killing assay with PBMCs might be majorly attributed to NK cells, tumor growth control *in vivo* in a 6-week experiment might be a composite effect of 'effector lymphocytes'. It remains unanswered if the difference observed in tumor growth control was exclusively due to suppression in NK-cell activity. Contributions of other effector lymphocytes like CD8+ cytotoxic T-cells could have very well played a role,

however minute, in tumor control. Future experiments involving depletion of cells of interest could answer these questions.

Survivability was comparable between groups. While 3/8 mice died in TUMOR control group due to tumor burden, only 2/8 mice in TUMOR+SMG PBMCs group and 1/8 TUMOR+1G PBMCs died during the experiment (fig. 19c). These events reflect the tumor burden observed in these groups validating the tumor control capacity reflected with general health of the animals, at least during the span of the experiment.

Clinical evaluation of GVHD also showed similar onset, progression and severity patterns between the groups. Animals started showing signs of GVHD around 2 weeks with ruffled fur and hunched posture at rest usually being the initial signs (fig. 19d). Over the duration of the experiment, progression to moderate GVHD showed clinical signs of reduced activity, ruffling of fur, extremely hunched posture during movement, and some signs of skin distress with loss of hair and scaling. Weight loss was also considered a sign of GVHD or general morbidity due to both tumor and pathophysiology of GVHD causing loss of weight and reduced food intake in the animal. GVHD did not progress to severe GVHD (>7) during the span of the experiment to estimate if it induced any graft-versus-leukemia (GVL) effects. While αβ-T cell activity is crucial to GVHD pathophysiology, this does not seem to be affected in our current model. Past evidence shows *in vitro* T-cell activity is suppressed after exposure to SMG (Hauschild et al., 2014). However, in our *in vivo* model, there might be a recovery of the lost function over the duration of the experiment. Since GVHD does not start showing clinical symptoms until 3-4 weeks into the experiment, this might reflect in similar GVHD onset patterns between the groups. While similar recovery is possible in NK cells too, failure to control tumor growth in

the initial weeks might be contributing to tumor burden reaching an uncontrollable level before NK cells regain their activity. The more efficient 1G-exposed PBMCs could be more adept at killing K562 tumor cells *in vivo* during the initial weeks, which might have resulted in stunted growth of the tumor in the later weeks.

Exposure to SMG did not impair immune cell ability to engraft *in vivo.* CD45h+ cells engrafted successfully after exposure to SMG with their engraftment dynamics mirroring that of immune cells exposed to 1G-control (fig. 21). NK cell proportions and absolute numbers did not differ between the groups. These findings alleviated concerns that exposure to SMG might hinder immune cells' ability to thrive *in vivo* which might have confounded our BLI findings. Initial weeks showed low CD45h+%s averaging around 1-5%, with most of the human cells in the peripheral blood being NK cells. Engraftment rose to higher levels reaching 20-30% at the end of the experiment (5 weeks after PBMCs injection) with drop in overall NK cell proportions. This coincided with onset and progression of GVHD since αβ-T cells are the main contributors to GVHD. Engraftment levels in mice that had tumor were also measured. There were no differences between the two groups with SMG-exposed PBMCs showing consistently comparable levels of CD45h+ and CD45h+CD3-CD56+ proportions. While blood levels of immune cells might not be reflective of engraftment levels in mice with tumor, this showed that human immune cells and NK cells thrived similarly in mice after exposure to 1G and SMG. *In vivo* expansion of (CD45h+CD3-CD56+) human NK cells in the initial weeks was to be expected in a NSG-tg(hu-IL15) mouse model. NSG-tg(hu-IL15) mice sustain IL-15 blood levels of 7.1 +/- 0.3 pg/ml (https://www.jax.org/strain/030890). These levels are considerably higher than the usual undetectable levels in human blood (≈1 pg/ml) (Bergamaschi et al.,

2012). This mouse model was precisely chosen to sustain and expand NK cells, since estimation of NK cell function *in vivo* was the underpinning question. NK cell levels drop in the later weeks when the differences in BLI between the groups start to be accentuated. It remains unclear if NK cells are exhausted in the later weeks or they traffic away from the peripheral blood.

In summary, hypothesis 2^6 holds true that exposure to SMG impaired anti-leukemia activity of human effector immune cells in an *in vivo* model.

In the third aim of this dissertation, subcutaneous IL2, a common immunotherapeutic strategy to enhance graft-versus-leukemia effect along with intra-peritoneal zoledronic acid, an amino-bisphosphonate were tested as countermeasures. IL-2 has been shown to accelerate NK cell reconstitution in T-cell depleted stem cell grafts, increase GVL without increasing GVHD, and improve outcomes in various cancer therapies. Additionally, zoledronic acid, an amino-bisphosphonate, already being considered as a spaceflight countermeasure (to prevent decreases in bone mineral density) has also been shown to increase γδ-T cell proportion and function. Here we report that systemic administration of ZOL+IL2 therapy improved anti-leukemia activity of SMG-exposed PBMCs *in vivo*.

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[6] Exposure to 12-hour SMG in a RCCS adversely affects anti-leukemia activity of human immune cells in vivo by impairing their ability to control tumor growth in a NSG-tg(hu-IL15) mice model.

While tumor growth rate of TUMOR+SMG PBMCs+ZOL+IL2 group was not different from TUMOR+1G PBMCs group, it was significantly lower than TUMOR+SMG PBMCs group (figure 23). When taken in context with the previous experiment showing significantly higher tumor growth rate after SMG exposure, these results suggest that ZOL+IL2 therapy improved anti-leukemia activity after SMG exposure. The peak BLI scores did not reach significance in this aim but there was a clear trend towards reduced peak BLI score in TUMOR+SMG+ZOL+IL2 group compared to TUMOR+SMG PBMCs group. There also appears to be a downward trend in BLI score for the TUMOR+SMG PBMCs+ZOL+IL2 group towards the end of the experiment, suggesting an improved and sustained anti-leukemia activity after ZOL+IL2 therapy.

Survivability was not altered because of ZOL+IL2 therapy. Only 1/6 mice died after ZOL+IL2 therapy in the duration of the experiment. This was due to the animal reaching sacrificial criteria for morbidity score due to weight loss and general malaise since the tumor translocated to the brain. Overall survivability was comparable to other PBMC groups suggesting that ZOL+IL2 therapy did not cause systemic toxic effect in the animals.

Engraftment levels did not change due to ZOL+IL2 therapy (figure 23). Non-significant increases in NK cell and γδ-T cell proportions were observed after ZOL+IL2 therapy as expected. Since NK cells and γδ-T cells do not contribute to GVHD pathophysiology, there were no differences in GVHD onset, progression and severity.

In summary, Hypothesis 3[7] holds true that ZOL+IL2 therapy improved anti-leukemia activity of human effector immune cells after exposure to SMG.

[7] Systemic administration of ZOL+IL-2 will boost the in vivo anti-leukemia capacity of human immune cells in an NSG-tg(hu-IL15) mice model and/or in an in vitro model.

Chapter 6

General Discussion

Immune dysregulation observed in spaceflight is a composite effect of plethora of hazardous stimuli to the human body. These include psychological and physiological stress, microgravity, radiation exposure, isolation and confinement, circadian disruption, sleep disturbances, to name a few. Understanding of the precise effect of each of these perturbations could help to devise appropriate countermeasures to preserve health, prevent disease and sustain human performance.

Due to paucity of previous research, we examined the effect of simulated microgravity on NK cells. We reported that NK cell function is suppressed after short-term exposure to SMG. NK cells exposed to SMG kill 50% fewer cancer cells. NK cells showed lower levels of cytotoxic granule expression, degranulation, and cytokine production after SMG exposure. Contrastingly, cancer cells exposed to SMG did not show increased susceptibility to NK cell lysis (Mylabathula et al., 2020). Continuing the work, in this dissertation we further examined if this *in vitro* loss of function will extend into a physiologically more relevant *in vivo* setting. Furthermore, we also explored the effect of SMG on other immune cells—namely γδ-T cells and CMV-specific T-cells—which are pertinent to immune health in spaceflight.

Main findings in this dissertation

Chapter 4:

1. Exposure to 12-hour SMG in a RCCS impaired *in vitro* expansion of CMV-specific T-cells. Exposure to expansion impaired their ability to later expand when stimulated with CMV peptides compared to 1G-control and 1G-rotational control but did not impair their ability to recognize CMV peptides.

2. Exposure to 12-hour SMG in a RCCS impaired *in vitro* expansion of γδ-T cells. Exposure to SMG lowered the yield of γδ-T cells at the end of expansion while not significantly altering the phenotype of the expansion product. Vγ9vδ2 and vγ9vδ2 proportions along with major activating receptors like NKG2D, NKp30 were similar to 1G-control while significant downregulation of inhibitory receptor CD158b was observed in the expansion product, after exposure to SMG. This downregulation of CD158b might have played a role in increased killing observed in the functional assays. When 1G-expanded γδ-T cells were exposed to SMG at the end of the expansion, there was significant upregulation of activating receptor NKG2D and a trend towards further downregulation of CD158b without concomitant increase in function. This might be due to saturation of NK receptor signaling that governs γδ-T cell response.

3. Exposure to 12-hour SMG in a RCCS did not impair function of CMV-specific T-cells. 1G-expanded cytotoxic CMV-specific T-cells killed comparable number of target cells (autologous PHA blasts pulsed with CMV-peptides) when exposed to 1G-control or SMG. Interestingly, SMG-exposed PBMCs that were later expanded in 1G to yield

CMV-specific T-cells killed more target cells compared to 1G-rotational control. It is possible that this increase in function was a result of altered cytotoxic capabilities in the expanded product, warranting further investigation.

4. Exposure to 12-hour SMG in a RCCS did not impair *in vitro* function of γδ-T cells. 1G-expanded γδ-T cells killed comparable number of K562 and U266 target cells after exposure to SMG. Significant upregulation of NKG2D and trending downregulation of CD158b after exposure to SMG did not result in increased killing capacity possibly due to NK receptor signaling saturation. Similar to viral specific T-cells, SMG-exposed PBMCs that were later expanded in 1G to yield γδ-T cells killed more U266 target cells but not more K562 target cells. Differential downregulation of inhibitory receptor CD158b in the expanded product might be responsible for increased activating signal resulting in increased killing.

Chapter 5:

1. Exposure to SMG impaired anti-leukemia activity of human immune cells *in vivo.* Human PBMCs exposed to SMG showed reduced capacity to control tumor growth rate *in vivo* compared to 1G-exposed PBMCs. While SMG exposed PBMCs showed some anti-leukemia activity, they did not significantly curtail tumor burden resulting in a growth rate that was comparable to TUMOR control. In contrast, 1G-exposed PBMCs significantly corralled tumor growth resulting in relatively stunted growth rate. Mice that received SMG exposed PBMCs also showed similar peak BLI scores to mice that received tumor cells only further underscoring their

inability to restrict tumor growth.

2. Exposure to SMG did not impair human immune cell ability to thrive *in vivo.* Human immune cell (CD45h+) engraftment levels were comparable in 1G and SMG exposed conditions. NK cell engraftment (CD45h+CD3-CD56+%) showed high proportions in initial weeks, as expected in a NSG-tg(hu-IL15) mouse model and steadily declined thereafter as prevalence of other subsets increased in peripheral circulation.

3. Onset, progression and severity of graft-versus-host disease was similar between the groups of mice that received 1G and SMG exposed PBMCs suggesting that αβ-T cells retained their ability to attack the host in a xenograft. GVHD was not allowed to progress beyond moderate levels to prevent resultant immunomodulatory effects and morbidity from confounding the graft-versus-leukemia effect.

4. Survival of mice did not differ between the groups that received 1G or SMG exposed PBMCs. Most of the mice that received PBMCs survived the duration of the experiment alleviating concerns that differential survival might confound graft-versus-leukemia results. GVHD and resulting morbidity also did not reach sacrificial criteria for the animals. As expected, TUMOR control mice showed higher mortality rate in the duration of experiment in accordance with their tumor

burden.

5. ZOL+IL2 therapy improved anti-leukemia activity of human immune cells *in vivo*. Mice that received SMG-exposed PBMCs and weekly ZOL+IL2 therapy showed improved control of tumor growth. At the end of the experiment, tumor burden in the group that received SMG PBMCs and ZOL+IL2 therapy was at levels comparable to mice that received 1G PBMCs. This suggested that the countermeasure effectively abrogated SMG induced suppression of function *in vivo*. These mice also showed similar peak BLI levels to mice that received 1G exposed PBMCs. Mice that received SMG exposed PBMCs but did not receive ZOL+IL2 therapy showed poorer control of tumor growth and higher peak BLI scores compared to both TUMOR+SMG PBMCs+ZOL+IL2 and TUMOR+1G PBMCs groups.

6. While ZOL+IL2 therapy did not show significant improvements in overall engraftment dynamics, there were trending increases in NK cell and γδ-T cell proportions in the peripheral blood suggesting that the improved tumor growth control to be driven by NK cells and γδ-T cells.

7. ZOL+IL2 therapy did not affect survival of the mice. The treatment also did not influence the onset, progression and severity of GVHD. This suggested that the ZOL+IL2 therapy did not collaterally increase αβ-T cell activity.

Limitations of new knowledge obtained and suggestions for future directions

1. In vitro exposure to SMG might not accurately reflect *in vivo* physiology. Our model improved the understanding of the effects of microgravity on immune cells after short-term exposure. How continued exposure to microgravity over a period of months, if not years, affects immune cells remains to be explored.

2. While NK cells remain crucial to containing systemic and localized tumor transformation, their function in controlling tumor depends on their ability to expand when receiving accurate stimulus, homing to the site of interest and tumor infiltration (in case of solid tumors). These functions need to be accurately modeled *in vivo* using solid tumor models.

3. There is also evidence that microgravity alters pathogen virulence (C A Nickerson et al., 2000). A conglomerate model that evaluates pathogen-immune cell interaction and their perturbations in microgravity will improve our understanding of the magnitude of this effect. 3D organoids could be used in models to examine changes in tumor infiltration by effector cells like NK cells (Barrila et al., 2010).

4. Lowered γδ-T cell and CMV-specific T-cell expansion potential was observed in these experiments. While microgravity did not affect the function of these cells *in vitro,* if lowered their ability to expand translates to *in vivo* remains to be explored in future studies. Their ability to expand *in vivo* will be crucial when the host is challenged with

a latent viral reactivation or a tumor transformation. Current ISS data suggests that spaceflight induced latent viral reactivation is not controlled. Our *in vivo* experiments showed non-significant increases in γδ-T cell proportions in response to systemic zoledronic acid administration. This suggests that γδ-T cell expansion due to zoledronic administration in crewmembers to preserve bone mineral density might have some collateral immune benefits.

5. Our *in vivo* experiments showed that exposure to SMG impaired anti-leukemia activity of human effector immune cells. This loss of function was regained after systemic ZOL+IL2 administration. We believe this to be an NK-cell mediated mechanism, drawing the conclusion from a combination of experiments showing *in vitro* loss of function of NK cells after SMG exposure, while no such loss was observed in other immune subsets like γδ-T cells. Regaining lost anti-leukemia activity after ZOL+IL2 therapy could be a combined effect of improved NK cell and γδ-T cell function. NK-cell or γδ-T cell depletion experiments, which would remove cells of interest from the PBMC inoculum and observe differences in anti-leukemia activity, would provide definitive answers to these questions.

6. It is unclear how much of the *in vivo* anti-leukemia activity observed in these experiments could be attributed to other effector cells like CD8+ T-cells. There is substantial amount of evidence connecting GVHD with GVL. Moderate to severe GVHD provides some GVL effect in allogeneic stem cell transplant recipients. Therefore, some of the anti-leukemia activity (at least in later weeks) in our *in vivo*

experiments could be attributed to allogeneic αβ-T cells that are sensitized against host antigens. Similar GVHD scores were observed between conditions (TUMOR+SMG PBMCs and TUMOR+1G PBMCs) in our experiments. This would mean that any GVL effect afforded by GVHD could also be similar between the groups, alleviating concerns of this GVL effect being a confounder. Additionally, general trends of tumor growth in our experiments do not show reduction of tumor burden after it reaches levels of $1e^8$-$1e^9$ photons/second, which is around week 4 of our experiment. This is when GVHD scores reach 4-5, contributing to some GVL effect. Therefore, this additional GVL effect might be insignificant to the general trend of tumor growth. One strategy to control this would be to administer anti-GVHD drugs like alemtuzumab (anti-CD52 monoclonal antibody) to deplete T-cell populations *in vivo* to prevent GVHD.

Conclusion

Exposure to microgravity detrimentally affects anti-leukemia activity of human immune cells *in vivo.* Natural killer cell function and expansion potential of other effector cells—namely γδ-T cells, CMV-specific T cells—appear to be detrimentally affected by microgravity. While exposure to SMG in a bioreactor vessel affords a glimpse into the effect of microgravity on immune cells, the overall effect of microgravity or spaceflight on the human immune system will also depend on other perturbations of spaceflight on the human body. Immune system will be a responder to the detrimental effect of various factors like exposure to galactic cosmic radiation, physiological and psychological stress of the mission, demands of the body during spaceflight and the coping mechanisms to altered nutrition, microbiome and circadian rhythms. While it is important to know various effects of SMG on several immune subsets, how these cells behave when exposed to a myriad of factors remains to be explored. In the words of neuroscientist, V. S. Ramachandran, "nature is not a linear phenomenon". Exposure to microgravity for prolonged periods might exponentially worsen human physiology or hasten their evolution to adapt. Predictive models that simultaneously include multiple hazardous stimuli and employ a systems physiology approach to study various organ systems would greatly benefit space life sciences research in the future. Future spacefarers should include scientists and physicians that could bring back invaluable data collected from zero gravity and partial gravities of lunar and Martian surfaces.

References

Acharya, M. M., Baulch, J. E., Klein, P. M., Baddour, A. A. D., Apodaca, L. A., Kramár, E. A., … Limoli, C. L. (2019). New concerns for neurocognitive function during deep space exposures to chronic, low dose-rate, neutron radiation. *ENeuro*, *6*(4), 1–15. https://doi.org/10.1523/ENEURO.0094-19.2019

Ade, C. J., Broxterman, R. M., & Barstow, T. J. (2015). VO(2max) and Microgravity Exposure: Convective versus Diffusive O(2) Transport. *Medicine and Science in Sports and Exercise*, *47*(7), 1351–1361. https://doi.org/10.1249/MSS.0000000000000557

Agha, N. H., Baker, F. L., Kunz, H. E., Spielmann, G., Mylabathula, P. L., Rooney, B. V., … Simpson, R. J. (2020). Salivary antimicrobial proteins and stress biomarkers are elevated during a 6-month mission to the International Space Station. *Journal of Applied Physiology*, *128*(2), 264–275. https://doi.org/10.1152/japplphysiol.00560.2019

Agha, N. H., Mehta, S. K., Rooney, B. V., Laughlin, M. S., Markofski, M. M., Pierson, D. L., … Simpson, R. J. (2020). Exercise as a countermeasure for latent viral reactivation during long duration space flight. *FASEB Journal*, *34*(2), 2869–2881. https://doi.org/10.1096/fj.201902327R

Aldinucci, D., Borghese, C., & Casagrande, N. (2019). Formation of the immunosuppressive microenvironment of classic hodgkin lymphoma and therapeutic approaches to counter it. *International Journal of Molecular Sciences*, *20*(10), 1–23. https://doi.org/10.3390/ijms20102416

Aleshcheva, G., Bauer, J., Hemmersbach, R., Slumstrup, L., Wehland, M., Infanger, M., & Grimm, D. (2016). Scaffold-free Tissue Formation Under Real and Simulated

Microgravity Conditions. *Basic & Clinical Pharmacology & Toxicology*, *119 Suppl*, 26–33. https://doi.org/10.1111/bcpt.12561

Antonsen, E, Bayuse, T, et al (NASA). (2017). Risk of Adverse Health Outcomes performance Decrements in Performance due to In-Flight Medical Conditions. *NASA Human Research Programme, Exploration Medical Capabilities Element*, 1–80. Retrieved from https://humanresearchroadmap.nasa.gov/evidence/reports/Medical.PDF

Bajgain, P., Mucharla, R., Wilson, J., Welch, D., Anurathapan, U., Liang, B., … Vera, J. F. (2014). Optimizing the production of suspension cells using the G-Rex "M" series. *Molecular Therapy. Methods & Clinical Development*, *1*, 14015. https://doi.org/10.1038/mtm.2014.15

Balthesen, M., Messerle, M., & Reddehase, M. J. (1993). Lungs are a major organ site of cytomegalovirus latency and recurrence. *Journal of Virology*, *67*(9), 5360–5366. https://doi.org/10.1128/jvi.67.9.5360-5366.1993

Barrila, J., Radtke, A. L., Crabbé, A., Sarker, S. F., Herbst-Kralovetz, M. M., Ott, C. M., & Nickerson, C. A. (2010). Organotypic 3D cell culture models: using the rotating wall vessel to study host-pathogen interactions. *Nature Reviews. Microbiology*, *8*(11), 791–801. https://doi.org/10.1038/nrmicro2423

Bate, S. L., Dollard, S. C., & Cannon, M. J. (2010). Cytomegalovirus Seroprevalence in the United States: The National Health and Nutrition Examination Surveys, 1988–2004. *Clinical Infectious Diseases*, *50*(11), 1439–1447. https://doi.org/10.1086/652438

Bergamaschi, C., Bear, J., Rosati, M., Beach, R. K., Alicea, C., Sowder, R., … Pavlakis, G. N. (2012). Circulating IL-15 exists as heterodimeric complex with soluble IL-15Rα in

human and mouse serum. *Blood*, *120*(1), e1–e8. https://doi.org/10.1182/blood-2011-10-384362

Bigley, A. B., Agha, N. H., Baker, F. L., Spielmann, G., Kunz, H. E., Mylabathula, P. L., … Simpson, R. J. (2018). NK-cell function is impaired during long-duration spaceflight. *Journal of Applied Physiology (Bethesda, Md. : 1985)*. https://doi.org/10.1152/japplphysiol.00761.2018

Bigley, A. B., Agha, N. H., Baker, F. L., Spielmann, G., Kunz, H. E., Mylabathula, P. L., … Simpson, R. J. (2019). NK cell function is impaired during long-duration spaceflight, 842–853. https://doi.org/10.1152/japplphysiol.00761.2018

Bigley, A. B., Spielmann, G., Agha, N., O'Connor, D. P., & Simpson, R. J. (2016). Dichotomous effects of latent CMV infection on the phenotype and functional properties of CD8+ T-cells and NK-cells. *Cellular Immunology*, *300*, 26–32. https://doi.org/10.1016/j.cellimm.2015.11.005

BIOMEDICAL RESULTS OF SKYLAB. (2013).

Biron, C. A., Byron, K. S., & Sullivan, J. L. (1989). Severe Herpesvirus Infections in an Adolescent without Natural Killer Cells. *New England Journal of Medicine*, *320*(26), 1731–1735. https://doi.org/10.1056/NEJM198906293202605

Blue, R. S., Chancellor, J. C., Suresh, R., Carnell, L. S., Reyes, D. P., Nowadly, C. D., & Antonsen, E. L. (2019). Challenges in Clinical Management of Radiation-Induced Illnesses During Exploration Spaceflight. *Aerospace Medicine and Human Performance*, *90*(11), 966–977. https://doi.org/10.3357/AMHP.5370.2019

Bosma, G. C., Custer, R. P., & Bosma, M. J. (1983). A severe combined immunodeficiency mutation in the mouse. *Nature*, *301*(5900), 527–530. https://doi.org/10.1038/301527a0

Brainard, G. C., Barger, L. K., Soler, R. R., & Hanifin, J. P. (2016). The development of lighting countermeasures for sleep disruption and circadian misalignment during spaceflight. *Current Opinion in Pulmonary Medicine*, *22*(6), 535–544. https://doi.org/10.1097/MCP.0000000000000329

Brehm, M. A., Aryee, K.-E., Bruzenksi, L., Greiner, D. L., Shultz, L. D., & Keck, J. (2018). Transgenic expression of human IL15 in NOD-scid IL2rgnull (NSG) mice enhances the development and survival of functional human NK cells. *The Journal of Immunology*, *200*(1 Supplement), 103.20 LP-103.20. Retrieved from http://www.jimmunol.org/content/200/1_Supplement/103.20.abstract

Britten, R. A., Davis, L. K., Jewell, J. S., Miller, V. D., Hadley, M. M., Sanford, L. D., … Lonart, G. (2014). Exposure to mission relevant doses of 1 GeV/Nucleon (56)Fe particles leads to impairment of attentional set-shifting performance in socially mature rats. *Radiation Research*, *182*(3), 292–298. https://doi.org/10.1667/RR3766.1

Buchheim, J. I., Hoskyns, S., Moser, D., Han, B., Deindl, E., Hörl, M., … Choukèr, A. (2018). Oxidative burst and Dectin-1-triggered phagocytosis affected by norepinephrine and endocannabinoids: Implications for fungal clearance under stress. *International Immunology*, *30*(2), 79–89. https://doi.org/10.1093/intimm/dxy001

Buchheim, J., Matzel, S., Rykova, M., Vassilieva, G., Ponomarev, S., Nichiporuk, I., … Choukèr, A. (2019). Stress Related Shift Toward Inflammaging in Cosmonauts After Long-Duration Space Flight, *10*(February), 1–13.

https://doi.org/10.3389/fphys.2019.00085

Buravkova, L. B., Rykova, M. P., Grigorieva, V., & Antropova, E. (2004). Cell interactions in microgravity: cytotoxic effects of natural killer cells in vitro. Journal of Gravitational Physiology Vol 11 (2).

Caccamo, N., Meraviglia, S., Scarpa, F., La Mendola, C., Santini, D., Bonanno, C. T., … Salerno, A. (2008). Aminobisphosphonate-activated gammadelta T cells in immunotherapy of cancer: doubts no more. *Expert Opinion on Biological Therapy*, *8*(7), 875–883. https://doi.org/10.1517/14712598.8.7.875

Cao, D., Song, J., Ling, S., Niu, S., Lu, L., Cui, Z., … Li, Y. (2019). Hematopoietic stem cells and lineage cells undergo dynamic alterations under microgravity and recovery conditions. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *33*(6), 6904–6918. https://doi.org/10.1096/fj.201802421RR

Cao, X., Shores, E. W., Hu-Li, J., Anver, M. R., Kelsail, B. L., Russell, S. M., … Leonard, W. J. (1995). Defective lymphoid development in mice lacking expression of the common cytokine receptor γ chain. *Immunity*, *2*(3), 223–238. https://doi.org/10.1016/1074-7613(95)90047-0

Cavanagh, P. R., Licata, A. A., & Rice, A. J. (2005). Exercise and pharmacological countermeasures for bone loss during long-duration space flight. *Gravitational and Space Biology Bulletin : Publication of the American Society for Gravitational and Space Biology*, *18*(2), 39–58.

Cekanaviciute, E., Rosi, S., & Costes, S. V. (2018). Central Nervous System Responses to

Simulated Galactic Cosmic Rays. *International Journal of Molecular Sciences*, *19*(11). https://doi.org/10.3390/ijms19113669

Chancellor, J. C., Scott, G. B. I., & Sutton, J. P. (2014). Space Radiation: The Number One Risk to Astronaut Health beyond Low Earth Orbit. *Life (Basel, Switzerland)*, *4*(3), 491–510. https://doi.org/10.3390/life4030491

Cheever, M. A., Greenberg, P. D., Fefer, A., & Gillis, S. (1982). Augmentation of the anti-tumor therapeutic efficacy of long-term cultured T lymphocytes by in vivo administration of purified interleukin 2. *The Journal of Experimental Medicine*, *155*(4), 968–980. https://doi.org/10.1084/jem.155.4.968

Chiossone, L., Dumas, P.-Y., Vienne, M., & Vivier, E. (2018). Natural killer cells and other innate lymphoid cells in cancer. *Nature Reviews. Immunology*, *18*(11), 671–688. https://doi.org/10.1038/s41577-018-0061-z

Cho, B., Spratford, C. M., Yoon, S., Cha, N., Banerjee, U., & Shim, J. (n.d.). chemosensation in Drosophila. *Nature Communications*, (2018), 1–12. https://doi.org/10.1038/s41467-018-04990-3

Choukèr, a, Thiel, M., Baranov, V., Meshkov, D., Kotov, a, Peter, K., … Christ, F. (2001). Simulated microgravity, psychic stress, and immune cells in men: observations during 120-day 6 degrees HDT. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *90*(5), 1736–1743.

Cook, C. H. (2007). Cytomegalovirus Reactivation in “Immunocompetent” Patients: A Call for Scientific Prophylaxis. *The Journal of Infectious Diseases*, *196*(9), 1273–1275. https://doi.org/10.1086/522433

Cooper, M. A., Bush, J. E., Fehniger, T. A., VanDeusen, J. B., Waite, R. E., Liu, Y., … Caligiuri, M. A. (2002). In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells. *Blood*, *100*(10), 3633–3638. https://doi.org/10.1182/blood-2001-12-0293

Correia, D. V, Lopes, A., & Silva-Santos, B. (2013). Tumor cell recognition by γδ T lymphocytes: T-cell receptor vs. NK-cell receptors. *Oncoimmunology*, *2*(1), e22892–e22892. https://doi.org/10.4161/onci.22892

Crucian, B. E., Choukèr, A., Simpson, R. J., Mehta, S., Marshall, G., Smith, S. M., … Sams, C. (2018). Immune system dysregulation during spaceflight: Potential countermeasures for deep space exploration missions. *Frontiers in Immunology*, *9*(JUN), 1–21. https://doi.org/10.3389/fimmu.2018.01437

Crucian, B. E., Stowe, R. P., Pierson, D. L., & Sams, C. F. (2008a). Immune system dysregulation following short- vs long-duration spaceflight. *Aviation, Space, and Environmental Medicine*, *79*(9), 835–843. https://doi.org/10.3357/asem.2276.2008

Crucian, B. E., Stowe, R. P., Pierson, D. L., & Sams, C. F. (2008b). Immune system dysregulation following short- vs long-duration spaceflight. *Aviation Space and Environmental Medicine*, *79*(9), 835–843. https://doi.org/10.3357/ASEM.2276.2008

Crucian, B., Kunz, H., & Sams, C. F. (n.d.). *Risk of Crew Adverse Health Event Due to Altered Immune Response Human Research Program Human Health Countermeasures Element*. Retrieved from https://ntrs.nasa.gov/search.jsp?R=20150008177

Crucian, B., & Sams, C. (2009). Immune system dysregulation during spaceflight: clinical risk for exploration-class missions. *Journal of Leukocyte Biology*, *86*(5), 1017–1018.

https://doi.org/10.1189/jlb.0709500

Crucian, B., Simpson, R. J., Mehta, S., Stowe, R., Chouker, A., Hwang, S. A., … Sams, C. (2014a). Terrestrial stress analogs for spaceflight associated immune system dysregulation. *Brain, Behavior, and Immunity*, *39*, 23–32. https://doi.org/10.1016/j.bbi.2014.01.011

Crucian, B., Simpson, R. J., Mehta, S., Stowe, R., Chouker, A., Hwang, S., … Sams, C. (2014b). Brain , Behavior , and Immunity Terrestrial stress analogs for spaceflight associated immune system dysregulation. *Brain Behavior and Immunity*, *39*, 23–32. https://doi.org/10.1016/j.bbi.2014.01.011

Crucian, B., Stowe, R., Mehta, S., Uchakin, P., Quiriarte, H., Pierson, D., & Sams, C. (2013). Immune system dysregulation occurs during short duration spaceflight on board the space shuttle. *Journal of Clinical Immunology*, *33*(2), 456–465. https://doi.org/10.1007/s10875-012-9824-7

Crucian, B., Stowe, R. P., Mehta, S., Quiriarte, H., Pierson, D., & Sams, C. (2015). Alterations in adaptive immunity persist during long-duration space fl ight, (January), 1–10. https://doi.org/10.1038/npjmgrav.2015.13

Crucian, B., Stowe, R., Quiriarte, H., Pierson, D., & Sams, C. (2011). Monocyte phenotype and cytokine production profiles are dysregulated by short-duration spaceflight. *Aviation, Space, and Environmental Medicine*, *82*(9), 857–862. https://doi.org/10.3357/asem.3047.2011

Cucinotta, F. A. (2014). Space radiation risks for astronauts on multiple International Space Station missions. *PloS One*, *9*(4), e96099. https://doi.org/10.1371/journal.pone.0096099

Cucinotta, F. A., To, K., & Cacao, E. (2017). Predictions of space radiation fatality risk for exploration missions. *Life Sciences in Space Research, 13,* 1–11. https://doi.org/10.1016/j.lssr.2017.01.005

Degan, P., Sancandi, M., Zunino, A., Ottaggio, L., Viaggi, S., Cesarone, F., … Abbondandolo, A. (2005). Exposure of human lymphocytes and lymphoblastoid cells to simulated microgravity strongly affects energy metabolism and DNA repair. *Journal of Cellular Biochemistry*, *94*(3), 460–469. https://doi.org/10.1002/jcb.20302

Duda, J., Karimi, M., Negrin, R. S., & Contag, C. H. (2007). Methods for imaging cell fates in hematopoiesis. *Methods in Molecular Medicine, 134,* 17–34. https://doi.org/10.1007/978-1-59745-223-6_2

Fehniger, T. A., & Caligiuri, M. A. (2001). Interleukin 15: biology and relevance to human disease. *Blood*, *97*(1), 14–32. https://doi.org/10.1182/blood.V97.1.14

Felix, K., Wise, K., Manna, S., Yamauchi, K., Wilson, B. L., Thomas, R. L., … Ramesh, G. T. (2004). Altered cytokine expression in tissues of mice subjected to simulated microgravity. *Molecular and Cellular Biochemistry*, *266*(1–2), 79–85. https://doi.org/10.1023/B:MCBI.0000049136.55611.dd

Feuerecker, M., Crucian, B. E., Quintens, R., Buchheim, J. I., Salam, A. P., Rybka, A., … Choukèr, A. (2019). Immune sensitization during 1 year in the Antarctic high-altitude Concordia Environment. *Allergy: European Journal of Allergy and Clinical Immunology*, *74*(1), 64–77. https://doi.org/10.1111/all.13545

Fischer, C. L., Daniels, J. C., Levin, W. C., Kimzey, S. L., Cobb, E. K., & Ritzmann, S. E. (1972). Effects of the space flight environment on man's immune system. II.

Lymphocyte counts and reactivity. *Aerospace Medicine*, *43*(10), 1122–1125.

Fischer, C. L., Gill, C., Daniels, J. C., Cobb, E. K., Berry, C. A., & Ritzmann, S. E. (1972). Effects of the space flight environment on man's immune system. I. Serum proteins and immunoglobulins. *Aerospace Medicine*, *43*(8), 856–859. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/5056037

Fitzgerald, W., Chen, S., Walz, C., Zimmerberg, J., Margolis, L., & Grivel, J.-C. (2009). Immune suppression of human lymphoid tissues and cells in rotating suspension culture and onboard the International Space Station. *In Vitro Cellular & Developmental Biology. Animal*, *45*(10), 622–632. https://doi.org/10.1007/s11626-009-9225-2

Fleming, C., Morrissey, S., Cai, Y., & Yan, J. (2017). γδ T Cells: Unexpected Regulators of Cancer Development and Progression. *Trends in Cancer*, *3*(8), 561–570. https://doi.org/10.1016/j.trecan.2017.06.003

Fobelo Lozano, M. J., & Sanchez-Fidalgo, S. (2019). Adherence and preference of intravenous zoledronic acid for osteoporosis versus other bisphosphonates. *European Journal of Hospital Pharmacy : Science and Practice*, *26*(1), 4–9. https://doi.org/10.1136/ejhpharm-2017-001258

Foster, J. S., Wheeler, R. M., & Pamphile, R. (2014). Host-microbe interactions in microgravity: assessment and implications. *Life (Basel, Switzerland)*, *4*(2), 250–266. https://doi.org/10.3390/life4020250

Freed, L. E., Pellis, N., Searby, N., de Luis, J., Preda, C., Bordonaro, J., & Vunjak-Novakovic, G. (1999). Microgravity cultivation of cells and tissues. *Gravitational and Space Biology Bulletin : Publication of the American Society for Gravitational and*

Space Biology, *12*(2), 57–66. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/11541784

Gaffen, S. L., & Liu, K. D. (2004). Overview of interleukin-2 function , production and clinical applications, *28*. https://doi.org/10.1016/j.cyto.2004.06.010

Garcia-Lora, A., Algarra, I., & Garrido, F. (2003). MHC class I antigens, immune surveillance, and tumor immune escape. *Journal of Cellular Physiology*, *195*(3), 346–355. https://doi.org/10.1002/jcp.10290

Gerdemann, U., Keirnan, J. M., Katari, U. L., Yanagisawa, R., Christin, A. S., Huye, L. E., … Leen, A. M. (2009). Rapidly Generated Multivirus-specific Cytotoxic T Lymphocytes for the Prophylaxis and Treatment of Viral Infections. *Molecular Therapy*, *20*(8), 1622–1632. https://doi.org/10.1038/mt.2012.130

Gibson, C. R., Alexander, D. J., & Hamilton, D. R. (2012). Human Health Countermeasures Element Evidence Report Risk of Spaceflight-Induced Intracranial Hypertension and Vision Alterations. *Evidence Report*.

Glaser, R., Kiecolt-Glaser, J. K., Speicher, C. E., & Holliday, J. E. (1985). Stress, loneliness, and changes in herpesvirus latency. *Journal of Behavioral Medicine*, *8*(3), 249–260. https://doi.org/10.1007/bf00870312

Glaser, R., Kiecolt-Glaser, J. K., Stout, J. C., Tarr, K. L., Speicher, C. E., & Holliday, J. E. (1985). Stress-related impairments in cellular immunity. *Psychiatry Research*, *16*(3), 233–239. https://doi.org/10.1016/0165-1781(85)90111-8

Gleeson, M., Pyne, D. B., Austin, J. P., Lynn Francis, J., Clancy, R. L., McDonald, W. A., &

Fricker, P. A. (2002). Epstein-Barr virus reactivation and upper-respiratory illness in elite swimmers. *Medicine and Science in Sports and Exercise*, *34*(3), 411–417. https://doi.org/10.1097/00005768-200203000-00005

Goel, N., Bale, T. L., Epperson, C. N., Kornstein, S. G., Leon, G. R., Palinkas, L. A., … Dinges, D. F. (2014). Effects of sex and gender on adaptation to space: Behavioral health. *Journal of Women's Health*, *23*(11), 975–986. https://doi.org/10.1089/jwh.2014.4911

Goodwin, T. J., Prewett, T. L., Wolf, D. A., & Spaulding, G. F. (1993). Reduced shear-stress - A major component in the ability of mammalian-tissues to form 3-dimensional assemblies in simulated microgravity. *Journal of Cellular Biochemistry*, *51*(3), 301–311.

Goodwin, T. J., Schroeder, W. F., Wolf, D. A., & Moyer, M. P. (1993). Rotating-wall vessel coculture of small intestine as a prelude to tissue modeling: aspects of simulated microgravity. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)*, *202*(2), 181–192. https://doi.org/10.3181/00379727-202-43525

Grimm, D., Wehland, M., Pietsch, J., Aleshcheva, G., Wise, P., Van Loon, J., … Bauer, J. (2014). Growing tissues in real and simulated microgravity: New methods for tissue engineering. *Tissue Engineering - Part B: Reviews*, *20*(6), 555–566. https://doi.org/10.1089/ten.teb.2013.0704

Guéguinou, N., Huin-Schohn, C., Bascove, M., Bueb, J.-L., Tschirhart, E., Legrand-Frossi, C., & Frippiat, J.-P. (2009). Could spaceflight-associated immune system weakening

preclude the expansion of human presence beyond Earth's orbit? *Journal of Leukocyte Biology*, *86*(5), 1027–1038. https://doi.org/10.1189/jlb.0309167

Hales, N. W., Yamauchi, K., Alicea, a, Sundaresan, a, Pellis, N. R., & Kulkarni, a D. (2002). A countermeasure to ameliorate immune dysfunction in in vitro simulated microgravity environment: role of cellularnucleotide nutrition. *In Vitro Cellular & Developmental Biology. Animal*, *38*(4), 213–217. https://doi.org/10.1290/1071-2690(2002)038<0213:ACTAID>2.0.CO;2

Hamilton, E., Clay, T. M., & Blackwell, K. L. (2011). New perspectives on zoledronic acid in breast cancer: potential augmentation of anticancer immune response. *Cancer Investigation*, *29*(8), 533–541. https://doi.org/10.3109/07357907.2011.605413

Hammond, T. G., & Hammond, J. M. (2001). Optimized suspension culture: The rotating-wall vessel. *American Journal of Physiology - Renal Physiology*, *281*(1 50-1), 12–25. https://doi.org/10.1152/ajprenal.2001.281.1.f12

Hauschild, S., Tauber, S., Lauber, B., Thiel, C. S., Layer, L. E., & Ullrich, O. (2014). T cell regulation in microgravity - The current knowledge from in vitro experiments conducted in space, parabolic flights and ground-based facilities. *Acta Astronautica*, *104*(1), 365–377. https://doi.org/10.1016/j.actaastro.2014.05.019

Heininger, A., Jahn, G., Engel, C., Notheisen, T., Unertl, K., & Hamprecht, K. (2001). Human cytomegalovirus infections in nonimmunosuppressed critically ill patients. *Critical Care Medicine*, *29*(3), 541–547.

Hewitt, E. W. (2003). The MHC class I antigen presentation pathway: strategies for viral immune evasion. *Immunology*, *110*(2), 163–169. https://doi.org/10.1046/j.1365-

2567.2003.01738.x

Higginson, E. E., Galen, J. E., Levine, M. M., & Tennant, S. M. (2016). Microgravity as a biological tool to examine host–pathogen interactions and to guide development of therapeutics and preventatives that target pathogenic bacteria. *Pathogens and Disease*, *74*(8). https://doi.org/10.1093/femspd/ftw095

Himizu, K. S., Ields, R. C. F., & Iedlin, M. G. (1999). Systemic administration of interleukin 2 enhances the therapeutic efficacy of dendritic cell-based tumor vaccines, *96*(March), 2268–2273.

Hosie, L., Pachnio, A., Zuo, J., Pearce, H., Riddell, S., & Moss, P. (2017). Cytomegalovirus-Specific T Cells Restricted by HLA-Cw*0702 Increase Markedly with Age and Dominate the CD8+ T-Cell Repertoire in Older People . *Frontiers in Immunology* . Retrieved from https://www.frontiersin.org/article/10.3389/fimmu.2017.01776

Hsieh, C. L., Chao, P. D. L., & Fang, S. H. (2005). Morin sulphates/glucuronides enhance macrophage function in microgravity culture system. *European Journal of Clinical Investigation*, *35*(9), 591–596. https://doi.org/10.1111/j.1365-2362.2005.01551.x

Huyan, T., Li, Q., Yang, H., Jin, M. L., Zhang, M. J., Ye, L. J., … Yin, D. C. (2014). Protective effect of polysaccharides on simulated microgravity-induced functional inhibition of human NK cells. *Carbohydrate Polymers*, *101*(1), 819–827. https://doi.org/10.1016/j.carbpol.2013.10.021

Jaber, S., Chanques, G., Borry, J., Souche, B., Verdier, R., Perrigault, P.-F., & Eledjam, J.-J. (2005). Cytomegalovirus infection in critically ill patients: associated factors and consequences. *Chest*, *127*(1), 233–241. https://doi.org/10.1378/chest.127.1.233

Jessup, J. M., Goodwin, T. J., & Spaulding, G. (1993). Prospects For Use Of Microgravity-Based Bioreactors To Study 3- Dimensional Host Tumor Interactions In Human Neoplasia. *Journal Of Cellular Biochemistry*, *51*(3), 290–300.

Johnston, R. S., Dietlein, L. F., Berry, C. A., Parker, F., Vernon, J., & Larry, E. (1975). Biomedical Results of Apollo.

Joye, A., & Gonzales, J. A. (2018). Ocular manifestations of cytomegalovirus in immunocompetent hosts. *Current Opinion in Ophthalmology*, *29*(6), 535–542. https://doi.org/10.1097/ICU.0000000000000521

Kamin, H. S., & Kertes, D. A. (2017). Cortisol and DHEA in development and psychopathology. *Hormones and Behavior*, *89*, 69–85. https://doi.org/10.1016/j.yhbeh.2016.11.018

Kandarian, F., Sunga, G. M., Arango-Saenz, D., & Rossetti, M. (2017). A flow cytometry-based cytotoxicity assay for the assessment of human NK cell activity. *Journal of Visualized Experiments*, *2017*(126), 1–8. https://doi.org/10.3791/56191

Kast, J., Yu, Y., Seubert, C. N., Wotring, V. E., & Derendorf, H. (2017). Drugs in space: Pharmacokinetics and pharmacodynamics in astronauts. *European Journal of Pharmaceutical Sciences*, *109*(May), S2–S8. https://doi.org/10.1016/j.ejps.2017.05.025

Kataoka, S., Satoh, J., Fujiya, H., Toyota, T., Suzuki, R., Itoh, K., & Kumagai, K. (1983). Immunologic aspects of the nonobese diabetic (NOD) mouse. Abnormalities of cellular immunity. *Diabetes*, *32*(3), 247–253. https://doi.org/10.2337/diab.32.3.247

Kaufmann, I., Schachtner, T., Feuerecker, M., Schelling, G., Thiel, M., & Choukèr, a.

(2009). Parabolic flight primes cytotoxic capabilities of polymorphonuclear leucocytes in humans. *European Journal of Clinical Investigation*. https://doi.org/10.1111/j.1365-2362.2009.02136.x

Kaufmann, Ines, Feuerecker, M., Salam, A., Schelling, G., Thiel, M., & Choukèr, A. (2011). Adenosine A2A receptor modulates the oxidative stress response of primed polymorphonuclear leukocytes after parabolic flight. *Human Immunology*, *72*(7), 547–552. https://doi.org/10.1016/j.humimm.2011.03.021

Kaur, I., Simons, E. R., Castro, V. a., Mark Ott, C., & Pierson, D. L. (2004). Changes in neutrophil functions in astronauts. *Brain, Behavior, and Immunity*, *18*(5), 443–450. https://doi.org/10.1016/j.bbi.2003.10.005

Kaur, I., Simons, E. R., Castro, V. a., Ott, C. M., & Pierson, D. L. (2005). Changes in monocyte functions of astronauts. *Brain, Behavior, and Immunity*, *19*(6), 547–554. https://doi.org/10.1016/j.bbi.2004.12.006

Kelsen, J., Bartels, L. E., Dige, A., Hvas, C. L., Frings-Meuthen, P., Boehme, G., … Dahlerup, J. F. (2012). 21 Days head-down bed rest induces weakening of cell-mediated immunity - Some spaceflight findings confirmed in a ground-based analog. *Cytokine*, *59*(2), 403–409. https://doi.org/10.1016/j.cyto.2012.04.032

Kimpo, M. S., Oh, B., & Lee, S. (2019). The Role of Natural Killer Cells as a Platform for Immunotherapy in Pediatric Cancers. *Current Oncology Reports*, *21*(10), 93. https://doi.org/10.1007/s11912-019-0837-8

Kita, M., Yamamoto, T., Imanishi, J., & Fuse, A. (2004). Influence of gravity changes induced by parabolic flight on cytokine production in mouse spleen. *Journal of*

Gravitational Physiology : A Journal of the International Society for Gravitational Physiology, *11*(2), P67—8. Retrieved from http://europepmc.org/abstract/MED/16235419

Klarenbeek, P. L., Remmerswaal, E. B. M., ten Berge, I. J. M., Doorenspleet, M. E., van Schaik, B. D. C., Esveldt, R. E. E., … van Lier, R. A. W. (2012). Deep Sequencing of Antiviral T-Cell Responses to HCMV and EBV in Humans Reveals a Stable Repertoire That Is Maintained for Many Years. *PLoS Pathogens*, *8*(9). https://doi.org/10.1371/journal.ppat.1002889

Klaus, D. M. (2001). Clinostats and bioreactors. *Gravitational and Space Biology Bulletin : Publication of the American Society for Gravitational and Space Biology*, *14*(2), 55–64.

Kondo, M., Izumi, T., Fujieda, N., Kondo, A., Morishita, T., Matsushita, H., & Kakimi, K. (2011). Expansion of human peripheral blood γδ T cells using zoledronate. *Journal of Visualized Experiments*, *2*(55), 6–11. https://doi.org/10.3791/3182

Kulkarni, A. D., Yamauchi, K., Sundaresan, A., Ramesh, G. T., & Pellis, N. R. (2005). Countermeasure for space flight effects on immune system: nutritional nucleotides. *Gravitational and Space Biology Bulletin : Publication of the American Society for Gravitational and Space Biology*, *18*(2), 101–102.

Kumari, R., Singh, K. P., & DuMond, J. W. (2009). Simulated microgravity decreases DNA repair capacity and induces DNA damage in human lymphocytes. *Journal of Cellular Biochemistry*, *107*(4), 723–731. https://doi.org/10.1002/jcb.22171

Lai, H. Y., Chou, T. Y., Tzeng, C. H., & Lee, O. K. S. (2012). Cytokine profiles in various graft-versus-host disease target organs following hematopoietic stem cell

transplantation. *Cell Transplantation*, *21*(9), 2033–2045. https://doi.org/10.3727/096368912X653110

Lappa, M. (2003). Organic tissues in rotating bioreactors: fluid-mechanical aspects, dynamic growth models, and morphological evolution. *Biotechnology and Bioengineering*, *84*(5), 518–532. https://doi.org/10.1002/bit.10821

LaVoy, E. C. P., Bollard, C. M., Hanley, P. J., Blaney, J. W., O'Connor, D. P., Bosch, J. A., & Simpson, R. J. (2015). A single bout of dynamic exercise enhances the expansion of MAGE-A4 and PRAME-specific cytotoxic T-cells from healthy adults. *Exercise Immunology Review*, *21*, 144–153.

Lawand, M., Déchanet-Merville, J., & Dieu-Nosjean, M. C. (2017). Key features of gamma-delta T-cell subsets in human diseases and their immunotherapeutic implications. *Frontiers in Immunology*, *8*(JUN). https://doi.org/10.3389/fimmu.2017.00761

Lee, E. H., Ding, W., Kulkarni, A. D., & Granstein, R. D. (2005). Tumor growth and immune function in mice during hind-limb unloading. *Aviation, Space, and Environmental Medicine*, *76*(6), 536–540.

Li, M., Holmes, V., Ni, H., Sanzari, J. K., Romero-Weaver, A. L., Lin, L., … Weissman, D. (2015). Broad-spectrum antibiotic or G-CSF as potential countermeasures for impaired control of bacterial infection associated with an SPE Exposure during spaceflight. *PLoS ONE*, *10*(3), 1–15. https://doi.org/10.1371/journal.pone.0120126

Li, M., Holmes, V., Zhou, Y., Ni, H., Sanzari, J. K., Kennedy, A. R., & Weissman, D. (2014). Hindlimb suspension and SPE-like radiation impairs clearance of bacterial infections. *PLoS ONE*, *9*(1). https://doi.org/10.1371/journal.pone.0085665

Li, Q., Mei, Q., Huyan, T., Xie, L., Che, S., Yang, H., … Huang, Q. (2013). Effects of Simulated Microgravity on Primary Human NK Cells. *Astrobiology*, *13*(8), 703–714. https://doi.org/10.1089/ast.2013.0981

Liakopoulos, V., Leivaditis, K., Eleftheriadis, T., & Dombros, N. (2012). The kidney in space. *International Urology and Nephrology*, *44*(6), 1893–1901. https://doi.org/10.1007/s11255-012-0289-7

Licato, L. L., & Grimm, E. a. (1999). Multiple interleukin-2 signaling pathways differentially regulated by microgravity. *Immunopharmacology*, *44*(3), 273–279. https://doi.org/10.1016/S0162-3109(99)00123-X

Lieberman, N. A. P., DeGolier, K., Haberthur, K., Chinn, H., Moyes, K. W., Bouchlaka, M. N., … Crane, C. A. (2018). An uncoupling of canonical phenotypic markers and functional potency of Ex vivo-expanded natural killer cells. *Frontiers in Immunology*, *9*(FEB), 1–15. https://doi.org/10.3389/fimmu.2018.00150

Liu, H., Wang, S.-H., Chen, S.-C., Chen, C.-Y., Lo, J.-L., & Lin, T.-M. (2016). Immune modulation of CD4(+)CD25(+) regulatory T cells by zoledronic acid. *BMC Immunology*, *17*(1), 45. https://doi.org/10.1186/s12865-016-0183-7

Maffiuletti, N. A., Green, D. A., Vaz, M. A., & Dirks, M. L. (2019). Neuromuscular Electrical Stimulation as a Potential Countermeasure for Skeletal Muscle Atrophy and Weakness During Human Spaceflight. *Frontiers in Physiology*, *10*, 1031. https://doi.org/10.3389/fphys.2019.01031

Mahmoudpour, S. H., Jankowski, M., Valerio, L., Becker, C., Espinola-klein, C., Konstantinides, S., … Barco, S. (2019). Safety of low-dose subcutaneous recombinant

interleukin-2 : systematic review and meta- analysis of randomized controlled trials. *Scientific Reports*, (March), 1–9. https://doi.org/10.1038/s41598-019-43530-x

Manley, P., & Lelkes, P. I. (2006). A novel real-time system to monitor cell aggregation and trajectories in rotating wall vessel bioreactors. *Journal of Biotechnology*, *125*(3), 416–424. https://doi.org/10.1016/j.jbiotec.2006.03.030

Martinelli, L., Russomano, T., Dos Santos, M., Falcao, F., Bauer, M., MacHado, A., & Sundaresan, A. (2009). Effect of microgravity on immune cell viability and proliferation. *IEEE Engineering in Medicine and Biology Magazine*, *28*(4), 85–90. https://doi.org/10.1109/MEMB.2009.933572

Martinez, E. M., Yoshida, M. C., Candelario, T. L. T., Hughes-Fulford, M., Baqai, F., Gridley, D., … Beg, A. (2015). Spaceflight and simulated microgravity cause a significant reduction of key gene expression in early T-cell activation. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *308*(6), R480-8. https://doi.org/10.1152/ajpregu.00449.2014

Mazumder, A., & Rosenberg, S. A. (1984). Successful immunotherapy of natural killer-resistant established pulmonary melanoma metastases by the intravenous adoptive transfer of syngeneic lymphocytes activated in vitro by interleukin 2. *The Journal of Experimental Medicine*, *159*(2), 495–507. https://doi.org/10.1084/jem.159.2.495

Mehta, S. K., Crucian, B. E., Stowe, R. P., Simpson, R. J., Ott, C. M., Sams, C. F., & Pierson, D. L. (2013). Reactivation of latent viruses is associated with increased plasma cytokines in astronauts. *Cytokine*, *61*(1), 205–209. https://doi.org/10.1016/j.cyto.2012.09.019

Mehta, S. K., Laudenslager, M. L., Stowe, R. P., Crucian, B. E., Sams, C. F., & Pierson, D. L. (2014). Multiple latent viruses reactivate in astronauts during Space Shuttle missions. *Brain, Behavior, and Immunity*, *41*(1), 210–217. https://doi.org/10.1016/j.bbi.2014.05.014

Mehta, Satish K., Laudenslager, M. L., Stowe, R. P., Crucian, B. E., Feiveson, A. H., Sams, C. F., & Pierson, D. L. (2017). Latent virus reactivation in astronauts on the international space station. *Npj Microgravity*, *3*(1), 1–7. https://doi.org/10.1038/s41526-017-0015-y

Mehta, Satish K., Pierson, D. L., Cooley, H., Dubow, R., & Lugg, D. (2000). Epstein-Barr virus reactivation associated with diminished cell-mediated immunity in antarctic expeditioners. *Journal of Medical Virology*, *61*(2), 235–240. https://doi.org/10.1002/(SICI)1096-9071(200006)61:2<235::AID-JMV10>3.0.CO;2-4

Mei, M., Xiang, Z., Yang, J., & Xiang, R. (2019). Efficacy of zoledronic acid for prevention of bone loss in early-stage breast cancer patients receiving adjuvant therapy: A meta-analysis of 13 randomized controlled trials. *Current Problems in Cancer*, 100507. https://doi.org/10.1016/j.currproblcancer.2019.100507

Meropol, N. J., Porter, M., Blumenson, L. E., Lindemann, M. J., Perez, R. P., Vaickus, L., … Caligiuri, M. A. (1996). Daily subcutaneous injection of low-dose interleukin 2 expands natural killer cells in vivo without significant toxicity. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *2*(4), 669–677.

Monici, M., Fusi, F., Paglierani, M., Marziliano, N., Cogoli, A., Pratesi, R., & Bernabei, P. A. (2006). Modeled gravitational unloading triggers differentiation and apoptosis in

preosteoclastic cells. *Journal of Cellular Biochemistry*, *98*(1), 65–80. https://doi.org/10.1002/jcb.20747

Monkkonen, H., Ottewell, P. D., Kuokkanen, J., Monkkonen, J., Auriola, S., & Holen, I. (2007). Zoledronic acid-induced IPP/ApppI production in vivo. *Life Sciences, 81*(13), 1066–1070. https://doi.org/10.1016/j.lfs.2007.08.007

Moreels, M., States, U., Ulrike, T., Quintens, R., & Sams, C. F. (2019). Paper ID : 12410 From Earth Missions to Deep Space Exploration (05) (session not specified) Author : Dr . Matthias Feuerecker University of Munich , Germany , matthias.feuerecker@med.uni-muenchen.de Dr . Ines Kaufmann Dr . Alex Paddy Salam United King, (05), 5–6.

Moreno-villanueva, M., Feiveson, A. H., Krieger, S., Brinda, A. K., Scheven, G. Von, Bürkle, A., … Wu, H. (n.d.). Synergistic Effects of Weightlessness , Isoproterenol , and Radiation on DNA Damage Response and Cytokine Production in Immune Cells, 1–23. https://doi.org/10.3390/ijms19113689

Morey-Holton, E. R., & Globus, R. K. (2002). Hindlimb unloading rodent model: technical aspects. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *92*(4), 1367–1377. https://doi.org/10.1152/japplphysiol.00969.2001

Mulavara, A. P., Peters, B. T., Miller, C. A., Kofman, I. S., Reschke, M. F., Taylor, L. C., … Bloomberg, J. J. (2018). Physiological and Functional Alterations after Spaceflight and Bed Rest. *Medicine and Science in Sports and Exercise*, *50*(9), 1961–1980. https://doi.org/10.1249/MSS.0000000000001615

Muntasell, A., Ochoa, M. C., Cordeiro, L., Berraondo, P., Lopez-Diaz de Cerio, A., Cabo,

M., … Melero, I. (2017). Targeting NK-cell checkpoints for cancer immunotherapy. *Current Opinion in Immunology*, *45*, 73–81. https://doi.org/10.1016/j.coi.2017.01.003

Mylabathula, P. L., Li, L., Bigley, A. B., Markofski, M. M., Crucian, B. E., Mehta, S. K., … Simpson, R. J. (2020). Simulated microgravity disarms human NK-cells and inhibits anti-tumor cytotoxicity in vitro. *Acta Astronautica*, *174*(March), 32–40. https://doi.org/10.1016/j.actaastro.2020.03.023

Narici, M. V, & de Boer, M. D. (2011). Disuse of the musculo-skeletal system in space and on earth. *European Journal of Applied Physiology*, *111*(3), 403–420. https://doi.org/10.1007/s00421-010-1556-x

Naserian, S., Leclerc, M., Thiolat, A., Pilon, C., Le Bret, C., Belkacemi, Y., … Cohen, J. L. (2018). Simple, reproducible, and efficient clinical grading system for murine models of acute graft-versus-host disease. *Frontiers in Immunology*, *9*(JAN), 1–9. https://doi.org/10.3389/fimmu.2018.00010

Nickerson, C A, Ott, C. M., Mister, S. J., Morrow, B. J., Burns-Keliher, L., & Pierson, D. L. (2000). Microgravity as a novel environmental signal affecting Salmonella enterica serovar Typhimurium virulence. *Infection and Immunity*, *68*(6), 3147–3152. https://doi.org/10.1128/iai.68.6.3147-3152.2000

Nickerson, Cheryl A., Ott, C. M., Wilson, J. W., Ramamurthy, R., LeBlanc, C. L., Höner zu Bentrup, K., … Pierson, D. L. (2003). Low-shear modeled microgravity: A global environmental regulatory signal affecting bacterial gene expression, physiology, and pathogenesis. *Journal of Microbiological Methods*, *54*(1), 1–11. https://doi.org/10.1016/S0167-7012(03)00018-6

Nielsen, M. M., Witherden, D. A., & Havran, W. L. (2017). γδ T cells in homeostasis and host defence of epithelial barrier tissues. *Nature Reviews. Immunology*, *17*(12), 733–745. https://doi.org/10.1038/nri.2017.101

Nilsson, U. R., & Muller-Eberhard, H. J. (1967). Deficiency of the fifth component of complement in mice with an inherited complement defect. *The Journal of Experimental Medicine*, *125*(1), 1–16. https://doi.org/10.1084/jem.125.1.1

Orr, M. T., & Lanier, L. L. (2010). Natural killer cell education and tolerance. *Cell*, *142*(6), 847–856. https://doi.org/10.1016/j.cell.2010.08.031

Ottewell, P. D., Mönkkönen, H., Jones, M., Lefley, D. V, Coleman, R. E., & Holen, I. (2008). Antitumor Effects of Doxorubicin Followed by Zoledronic Acid in a Mouse Model of Breast Cancer, 1167–1178. https://doi.org/10.1093/jnci/djn240

Paley, M. A., & Prescher, J. A. (2014). Bioluminescence: a versatile technique for imaging cellular and molecular features. *MedChemComm*, *5*(3), 255–267. https://doi.org/10.1039/C3MD00288H

Parihar, V. K., Allen, B. D., Caressi, C., Kwok, S., Chu, E., Tran, K. K., … Limoli, C. L. (2016). Cosmic radiation exposure and persistent cognitive dysfunction. *Scientific Reports*, *6*, 34774. https://doi.org/10.1038/srep34774

Park, K.-H., Park, H., Kim, M., Kim, Y., Han, K., & Oh, E.-J. (2013). Evaluation of NK Cell Function by Flowcytometric Measurement and Impedance Based Assay Using Real-Time Cell Electronic Sensing System. *BioMed Research International*, *2013*, 210726. https://doi.org/10.1155/2013/210726

Paulsen, K., Thiel, C., Timm, J., Schmidt, P. M., Huber, K., Tauber, S., … Ullrich, O. (2010). Microgravity-induced alterations in signal transduction in cells of the immune system. *Acta Astronautica*, *67*(9–10), 1116–1125. https://doi.org/10.1016/j.actaastro.2010.06.053

Pauza, C. D., Liou, M.-L., Lahusen, T., Xiao, L., Lapidus, R. G., Cairo, C., & Li, H. (2018). Gamma Delta T Cell Therapy for Cancer: It Is Good to be Local. *Frontiers in Immunology*, *9*, 1305. https://doi.org/10.3389/fimmu.2018.01305

Pearson, T., Markees, T. G., Serreze, D. V., Pierce, M. A., Marron, M. P., Wicker, L. S., … Greiner, D. L. (2003). Genetic Disassociation of Autoimmunity and Resistance to Costimulation Blockade-Induced Transplantation Tolerance in Nonobese Diabetic Mice. *The Journal of Immunology*, *171*(1), 185–195. https://doi.org/10.4049/jimmunol.171.1.185

Pellis, N. R., Goodwin, T. J., Risin, D., Mcintyre, B. W., Pizzini, R. P., Cooper, D., … Spaulding, G. F. (1997). Changes in gravity inhibit lymphocyte locomotion through type I collagen. *In Vitro Cellular and Developmental Biology - Animal*, *33*(5), 398–405. https://doi.org/10.1007/s11626-997-0012-7

Pistoia, V., Tumino, N., Vacca, P., Veneziani, I., Moretta, A., Locatelli, F., & Moretta, L. (2018). Human γδ T-Cells: From surface receptors to the therapy of high-risk leukemias. *Frontiers in Immunology*, *9*(MAY). https://doi.org/10.3389/fimmu.2018.00984

Polić, B., Hengel, H., Krmpotić, A., Trgovcich, J., Pavić, I., Lučin, P., … Koszinowski, U. H. (1998). Hierarchical and redundant lymphocyte subset control precludes

cytomegalovirus replication during latent infection. *Journal of Experimental Medicine*, *188*(6), 1047–1054. https://doi.org/10.1084/jem.188.6.1047

Port, A. D., Orlin, A., Kiss, S., Patel, S., D'Amico, D. J., & Gupta, M. P. (2017). Cytomegalovirus Retinitis: A Review. *Journal of Ocular Pharmacology and Therapeutics : The Official Journal of the Association for Ocular Pharmacology and Therapeutics*, *33*(4), 224–234. https://doi.org/10.1089/jop.2016.0140

Pressey, J. G., Adams, J. M., Kelly, D. R., & Lamb, L. S. (2013). Trial of zoledronic acid and interleukin-2 to expand tumoricidal γδ T cells in vivo in patients with refractory neuroblastoma. *Journal of Clinical Oncology*, *31*(15_suppl), TPS10073–TPS10073. https://doi.org/10.1200/jco.2013.31.15_suppl.tps10073

Risso, A., Tell, G., Vascotto, C., Costessi, A., Arena, S., Scaloni, A., & Cosulich, M. E. (2005). Activation of human T lymphocytes under conditions similar to those that occur during exposure to microgravity: A proteomics study. *Proteomics*, *5*(7), 1827–1837. https://doi.org/10.1002/pmic.200401082

Rivera, C. A., Tcharmtchi, M. H., Mendoza, L., & Smith, C. W. (2003). Endotoxemia and hepatic injury in a rodent model of hindlimb unloading. *Journal of Applied Physiology*, *95*(4), 1656–1663. https://doi.org/10.1152/japplphysiol.00302.2003

Roberts, D. R., Albrecht, M. H., Collins, H. R., Asemani, D., Chatterjee, A. R., Spampinato, M. V., … Antonucci, M. U. (2017). Effects of spaceflight on astronaut brain structure as indicated on MRI. *New England Journal of Medicine*, *377*(18), 1746–1753. https://doi.org/10.1056/NEJMoa1705129

Rooney, B. V, Crucian, B. E., Pierson, D. L., Laudenslager, M. L., Mehta, S. K., Graham, P.,

& Kennedy, E. (2019). Herpes Virus Reactivation in Astronauts During Spaceflight and Its Application on Earth, *10*(February), 1–9. https://doi.org/10.3389/fmicb.2019.00016

Rosenberg, S. A. (2014). IL-2: the first effective immunotherapy for human cancer. *Journal of Immunology (Baltimore, Md. : 1950)*, *192*(12), 5451–5458. https://doi.org/10.4049/jimmunol.1490019

Sager, K., Alam, S., Bond, A., Chinnappan, L., & Probert, C. S. (2015). Review article: cytomegalovirus and inflammatory bowel disease. *Alimentary Pharmacology & Therapeutics*, *41*(8), 725–733. https://doi.org/10.1111/apt.13124

Salaroglio, I. C., Campia, I., Kopecka, J., Gazzano, E., Orecchia, S., Ghigo, D., & Riganti, C. (2015). Zoledronic acid overcomes chemoresistance and immunosuppression of malignant mesothelioma. *Oncotarget*, *6*(2), 1128–1142. https://doi.org/10.18632/oncotarget.2731

Sams, C. F., Crucian, B. E., Stowe, R. P., Mehta, S. K., Yetman, D. L., Leal, M. J., … Pierson, D. L. (2009). Immune status, latent viral reactivation, and stress during long-duration head-down bed rest. *Aviation Space and Environmental Medicine*, *80*(5 PART 2), 37–44. https://doi.org/10.3357/ASEM.BR05.2009

Sarkar, P., Sarkar, S., Ramesh, V., Kim, H., Barnes, S., Kulkarni, A., … Ramesh, G. T. (2008). Proteomic analysis of mouse hypothalamus under simulated microgravity. *Neurochemical Research*, *33*(11), 2335–2341. https://doi.org/10.1007/s11064-008-9738-1

Schwarz, R. P., Goodwin, T. J., & Wolf, D. A. (1992). Cell culture for three-dimensional modeling in rotating-wall vessels: An application of simulated microgravity. *Journal of*

Tissue Culture Methods, *14*(2), 51–57. https://doi.org/10.1007/BF01404744

Senatore, G., Mastroleo, F., Leys, N., & Mauriello, G. (2018). Effect of microgravity & space radiation on microbes. *Future Microbiology*, *13*, 831–847. https://doi.org/10.2217/fmb-2017-0251

Serreze, D. V., Gaedeke, J. W., & Leiter, E. H. (1993). Hematopoietic stem-cell defects underlying abnormal macrophage development and maturation in NOD/Lt mice: Defective regulation of cytokine receptors and protein kinase C. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(20), 9625–9629. https://doi.org/10.1073/pnas.90.20.9625

Shearer, W. T., Lugg, D. J., Rosenblatt, H. M., Nickolls, P. M., Sharp, R. M., Reuben, J. M., & Ochs, H. D. (2001). Antibody responses to bacteriophage φX-174 in human subjects exposed to the Antarctic winter-over model of spaceflight. *Journal of Allergy and Clinical Immunology*, *107*(1), 160–164. https://doi.org/10.1067/mai.2001.112269

Shen, M., & Frishman, W. H. (2019). Effects of Spaceflight on Cardiovascular Physiology and Health. *Cardiology in Review*, *27*(3), 122–126. https://doi.org/10.1097/CRD.0000000000000236

Shultz, L. D., Lyons, B. L., Burzenski, L. M., Gott, B., Chen, X., Chaleff, S., … Handgretinger, R. (2005). Human Lymphoid and Myeloid Cell Development in NOD/LtSz-scid IL2R null Mice Engrafted with Mobilized Human Hemopoietic Stem Cells. *The Journal of Immunology*, *174*(10), 6477–6489. https://doi.org/10.4049/jimmunol.174.10.6477

Shultz, Leonard D., Ishikawa, F., & Greiner, D. L. (2007). Humanized mice in translational

biomedical research. *Nature Reviews Immunology*, *7*(2), 118–130. https://doi.org/10.1038/nri2017

Siddiqi 1966-, A. A. (2000). *Challenge to Apollo : the Soviet Union and the space race, 1945-1974*. Washington, D.C. : National Aeronautics and Space Administration, NASA History Div., Office of Policy and Plans, 2000. Retrieved from https://search.library.wisc.edu/catalog/999949792102121

Sierich, H., & Eiermann, T. (2013). Comparing Individual NK Cell Activity In Vitro. *Current Protocols in Immunology*, *100*(1), 14.32.1-14.32.11. https://doi.org/10.1002/0471142735.im1432s100

Simons, D. M., Gardner, E. M., & Lelkes, P. I. (2009). Sub-mitogenic phorbol myristate acetate co-stimulation rescues the PHA-induced activation of both na??ve and memory T cells cultured in the rotating-wall vessel bioreactor. *Cell Biology International*, *33*(8), 882–886. https://doi.org/10.1016/j.cellbi.2009.04.024

Singh, K. P., Kumari, R., & DuMond, J. W. (2010). Simulated microgravity-induced epigenetic changes in human lymphocytes. *Journal of Cellular Biochemistry*, *111*(1), 123–129. https://doi.org/10.1002/jcb.22674

Smith, S. M., Heer, M., Shackelford, L. C., Sibonga, J. D., Spatz, J., Pietrzyk, R. A., … Zwart, S. R. (2015). Bone metabolism and renal stone risk during International Space Station missions. *Bone*, *81*, 712–720. https://doi.org/10.1016/j.bone.2015.10.002

Spielmann, G., Agha, N., Kunz, H., Simpson, R. J., Crucian, B., Mehta, S., … Campbell, J. (2019). B cell homeostasis is maintained during long-duration spaceflight. *Journal of Applied Physiology*, *126*(2), 469–476. https://doi.org/10.1152/japplphysiol.00789.2018

Spielmann, G., Bollard, C. M., Kunz, H., Hanley, P. J., & Simpson, R. J. (2016a). A single exercise bout enhances the manufacture of viral-specific T-cells from healthy donors : implications for allogeneic adoptive transfer immunotherapy. *Nature Publishing Group*, (January), 1–13. https://doi.org/10.1038/srep25852

Spielmann, G., Bollard, C. M., Kunz, H., Hanley, P. J., & Simpson, R. J. (2016b). A single exercise bout enhances the manufacture of viral-specific T-cells from healthy donors: Implications for allogeneic adoptive transfer immunotherapy. *Scientific Reports*, *6*(January), 1–13. https://doi.org/10.1038/srep25852

Stervbo, U., Roch, T., Kornprobst, T., Sawitzki, B., Grutz, G., Wilhelm, A., … Babel, N. (2018). Gravitational stress during parabolic flights reduces the number of circulating innate and adaptive leukocyte subsets in human blood. *PloS One*, *13*(11), e0206272. https://doi.org/10.1371/journal.pone.0206272

Stokes, J., Hoffman, E. A., Zeng, Y., Larmonier, N., & Katsanis, E. (2016). Post-transplant bendamustine reduces GvHD while preserving GvL in experimental haploidentical bone marrow transplantation. *British Journal of Haematology*. https://doi.org/10.1111/bjh.14034

Stowe, R. P., Pierson, D. L., & Barrett, A. D. T. (2001). Elevated stress hormone levels relate to Epstein-Barr virus reactivation in astronauts. *Psychosomatic Medicine*, *63*(6), 891–895. https://doi.org/10.1097/00006842-200111000-00007

Stowe, R. P., Sams, C. F., Mehta, S. K., Kaur, I., Jones, M. L., Feeback, D. L., & Pierson, D. L. (1999). Leukocyte subsets and neutrophil function after short-term spaceflight Abstract : Changes in leukocyte subpopulations and function after spaceflight have been

observed, *65*(February), 179–186. https://doi.org/10.1002/jlb.65.2.179

Stowe, R. P., Yetman, D. L., Storm, W. F., Sams, C. F., & Pierson, D. L. (2008). Neuroendocrine and immune responses to 16-day bed rest with realistic launch and landing G profiles. *Aviation, Space, and Environmental Medicine*, *79*(2), 117–122. https://doi.org/10.3357/asem.2205.2008

Strewe, C., Crucian, B. E., Sams, C. F., Feuerecker, B., Stowe, R. P., Choukèr, A., & Feuerecker, M. (2015). Hyperbaric hyperoxia alters innate immune functional properties during NASA Extreme Environment Mission Operation (NEEMO). *Brain, Behavior, and Immunity*, *50*, 52–57. https://doi.org/10.1016/j.bbi.2015.06.017

Strewe, Claudia, Feuerecker, M., Nichiporuk, I., Kaufmann, I., Hauer, D., Morukov, B., … Choukèr, A. (2012). Effects of parabolic flight and spaceflight on the endocannabinoid system in humans. *Reviews in the Neurosciences*, *23*(5–6), 673–680. https://doi.org/10.1515/revneuro-2012-0057

Sundaresan, A., Risin, D., & Pellis, N. R. (2002). Loss of signal transduction and inhibition of lymphocyte locomotion in a ground-based model of microgravity. *In Vitro Cellular & Developmental Biology. Animal*, *38*(2), 118–122. https://doi.org/10.1290/1071-2690(2002)038<0118:LOSTAI>2.0.CO;2

Swaffield, T. P., Neviaser, A. S., & Lehnhardt, K. (2018). Fracture Risk in Spaceflight and Potential Treatment Options. *Aerospace Medicine and Human Performance*, *89*(12), 1060–1067. https://doi.org/10.3357/AMHP.5007.2018

Sylvester, C. B., Abe, J.-I., Patel, Z. S., & Grande-Allen, K. J. (2018). Radiation-Induced Cardiovascular Disease: Mechanisms and Importance of Linear Energy Transfer.

Frontiers in Cardiovascular Medicine, *5*, 5. https://doi.org/10.3389/fcvm.2018.00005

Sylwester, A. W., Mitchell, B. L., Edgar, J. B., Taormina, C., Pelte, C., Ruchti, F., … Picker, L. J. (2005). Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. *Journal of Experimental Medicine, 202*(5), 673–685. https://doi.org/10.1084/jem.20050882

Thiel, C. S., Paulsen, K., Bradacs, G., Lust, K., Tauber, S., Dumrese, C., … Ullrich, O. (2012). Rapid alterations of cell cycle control proteins in human T lymphocytes in microgravity. *Cell Communication and Signaling, 10*(1), 1. https://doi.org/10.1186/1478-811x-10-1

Topham, N. J., & Hewitt, E. W. (2009). Natural killer cell cytotoxicity: how do they pull the trigger? *Immunology*, *128*(1), 7–15. https://doi.org/10.1111/j.1365-2567.2009.03123.x

Tsao, Y. D., Goodwin, T. L., Wolf, D. A., & Spaulding, G. F. (1992). Responses of Gravity Level Variations on the NASA/JSC Bioreactor System. *Physiologist*, (March 1992). Retrieved from file:///C:/Users/mikep/Downloads/Tsao Goodwin Wolf Spaulding 1992.pdf

van der Heiden, P., Marijt, E., Falkenburg, F., & Jedema, I. (2018). Control of Cytomegalovirus Viremia after Allogeneic Stem Cell Transplantation: A Review on CMV-Specific T Cell Reconstitution. *Biology of Blood and Marrow Transplantation*, *24*(9), 1776–1782. https://doi.org/10.1016/j.bbmt.2018.03.028

Van Ombergen, A., Jillings, S., Jeurissen, B., Tomilovskaya, E., Rühl, R. M., Rumshiskaya, A., … Wuyts, F. L. (2018, October). Brain Tissue-Volume Changes in Cosmonauts. *The New England Journal of Medicine*. United States.

https://doi.org/10.1056/NEJMc1809011

Van Ombergen, A., Jillings, S., Jeurissen, B., Tomilovskaya, E., Rumshiskaya, A., Litvinova, L., … Wuyts, F. L. (2019). Brain ventricular volume changes induced by long-duration spaceflight. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(21), 10531–10536. https://doi.org/10.1073/pnas.1820354116

Walther, I. (2001). Space bioreactors: their use, their future. *Journal of Gravitational Physiology : A Journal of the International Society for Gravitational Physiology*, *8*(1), P13–P16. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/12638605

Wilke, C. M., Wu, K., Zhao, E., Wang, G., & Zou, W. (2010). Prognostic significance of regulatory T cells in tumor. *International Journal of Cancer*, *127*(4), 748–758. https://doi.org/10.1002/ijc.25464

Williams, N. S., Klem, J., Puzanov, I. J., Sivakumar, P. V, Schatzle, J. D., Bennett, M., & Kumar, V. (1998). Natural killer cell differentiation: insights from knockout and transgenic mouse models and in vitro systems. *Immunological Reviews*, *165*(1), 47–61. https://doi.org/10.1111/j.1600-065X.1998.tb01229.x

Wood, S. J., Loehr, J. A., & Guilliams, M. E. (2011). Sensorimotor reconditioning during and after spaceflight. *NeuroRehabilitation*, *29*(2), 185–195. https://doi.org/10.3233/NRE-2011-0694

Wu, B., Wang, Y., Wu, X., Liu, D., Xu, D., & Wang, F. (2018). On-orbit sleep problems of astronauts and countermeasures. *Military Medical Research*, *5*(1), 17. https://doi.org/10.1186/s40779-018-0165-6

Wuest, S. L., Richard, S., Kopp, S., Grimm, D., & Egli, M. (2015). Simulated microgravity: Critical review on the use of random positioning machines for mammalian cell culture. *BioMed Research International*, *2015*. https://doi.org/10.1155/2015/971474

Xu, X., Li, P., Zhang, P., Chu, M., Liu, H., Chen, X., & Ge, Q. (2016). Differential effects of Rhodiola rosea on regulatory T cell differentiation and interferongamma production in vitro and in vivo. *Molecular Medicine Reports*, *14*(1), 529–536. https://doi.org/10.3892/mmr.2016.5278

Xu, X., Tan, C., Li, P., Zhang, S., Pang, X., Liu, H., … Ge, Q. (2013). Changes of Cytokines during a Spaceflight Analog - a 45-Day Head-Down Bed Rest. *PLoS ONE*, *8*(10), 1–12. https://doi.org/10.1371/journal.pone.0077401

Yamauchi, K., Hales, N. W., Robinson, S. M., Niehoff, M. L., Ramesh, V., Pellis, N. R., & Kulkarni, A. D. (2002). Dietary nucleotides prevent decrease in cellular immunity in ground-based microgravity analog. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *93*(1), 161–166. https://doi.org/10.1152/japplphysiol.01084.2001

Yu, X., Zheng, L., Xiong, S., Cai, Z., & Wang, Q. (2011). [Effect of simulated microgravity on human monocytic cell proliferation and tissue factor mRNA expression]. *Nan fang yi ke da xue xue bao = Journal of Southern Medical University*, *31*(6), 1020–1022.

Zhang, L.-F., & Hargens, A. R. (2018). Spaceflight-Induced Intracranial Hypertension and Visual Impairment: Pathophysiology and Countermeasures. *Physiological Reviews*, *98*(1), 59–87. https://doi.org/10.1152/physrev.00017.2016

Zhao, Y., Niu, C., & Cui, J. (2018). Gamma-delta (γδ) T Cells: Friend or Foe in Cancer Development. *Journal of Translational Medicine*, *16*(1), 1–13.

https://doi.org/10.1186/s12967-017-1378-2

Zheng, L., Liu, J.-Z., Hu, Y.-W., Zhong, T.-Y., Xiong, S.-L., Wang, W., & Wang, Q. (2011). Simulated microgravity, erythroid differentiation, and the expression of transcription factor GATA-1 in CD34+ cells. *Aviation, Space, and Environmental Medicine*, *82*(5), 513–517. https://doi.org/10.3357/asem.2838.2011

Zwart, S. R., Mehta, S. K., Ploutz-Snyder, R., Bourbeau, Y., Locke, J. P., Pierson, D. L., & Smith, S. M. (2011). Response to Vitamin D Supplementation during Antarctic Winter Is Related to BMI, and Supplementation Can Mitigate Epstein-Barr Virus Reactivation. *The Journal of Nutrition*, *141*(4), 692–697. https://doi.org/10.3945/jn.110.134742